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March 1979

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by

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INIS Descriptors

BIOLOGICAL RADIATION EFFECTS
CALCIUM
CARROTS
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GROWTH
HEALING
HYDROLASES
INHIBITION
IONIZING RADIATION EFFECTS
LYASES
MUSHROOMS
ONIONS
OXALIC ACID
PATHOGENESIS
PECTINS
PERMEABILITY
PLANT DISEASES
PLANT TISSUES
POTATOES
SIDE EFFECTS
SPROUT INHIBITION
WOUNDS

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Denne afhandling samt 11 tilsluttende arbejder er at Den kgl. Veterinær- og Landbohøjskoles fagråd for landbrugsvidenskab antaget til offentligt at forsvares for den jordbrugsvidenskabelige doktorgrad.

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ABBREVIATIONS

CD_{50} = 50% reduction in crushing load.

Ci* = curie; 1 Ci = $3.7 \cdot 10^{10}$ disintegrations per second.

CMC = carboxymethylcellulose.

DEAE = cellulose N,N-diethylaminoethyl-ether.

EDTA = ethylenediaminetetra-acetic acid.

PAL, endo-PAL, exo-PAL = pectate lyases which may split the pectate chain transeliminatively more or less at random (endo) or from the end (exo).

PE = pectinesterase.

PG, endo-PG, exo-PG = polygalacturonases that are more active in hydrolysis of pectate than of pectin. They hydrolyze the molecular chain more or less at random (endo) or from the end (exo).

PL, endo-PL, exo-PL = pectin lyases which differ from PAL (cf. this) in that they prefer pectin to pectate as substrate.

PMG, endo-PMG, exo-PMG = polymethylgalacturonases which differ from PG (cf. this) in that they prefer pectin to pectate as substrate.

p.p. = phloem parenchyma tissue between the cambial region and the cork/periderm layers.

r = roentgen. 1 r produces 2.1×10^9 ion pairs per cm^3 of air under standard conditions. This equals 83.8 erg of energy absorbed in 1 g of air (also gram-roentgen). Kr = 10^3 r.

rad* = 100 erg (10^{-2} joule/kg) energy absorbed per g of water or biological tissue. 1 krad = 10^3 rad; 1 Mrad = 10^6 rad.

rep (roentgen equivalent, physical) = 93 erg absorbed energy per g water or biological tissue, i.e. the same amount of energy released in water or tissue as 1 r of X-rays (compare above). 1 krep = 10^3 rep; 1 Mrep = 10^6 rep.

r.h. = relative humidity.

TBA = 2-thiobarbituric acid.

tris-= tris(hydroxymethyl)aminoethane used in buffers together with HCl or acetic acid.

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* The special unit Bq (bequerel) have replaced Ci (curie) and the special unit Gy (gray) have replaced rad in the SI system (Système International d'Unités). 1 Bq = 1 s^{-1} (1 disintegration per second) and 1 Gy = 10^2 rad (cf. Encyclopedia of Science and Technology, Vol. 15, p. LXIX - LXXXIII, 1977, Handelsministeriets bekendtgørelse nr. 320 af 21. maj 1977).

PREFACE

Shortly after the second world war considerable attention was paid to the application of ionizing radiation to food preservation because of optimism concerning a number of seemingly attractive possibilities. Most of the optimistic reports originated in the USA during the fifties, but there were also some from England and a little later from other European countries. Later, however, as the number of papers increased, optimism waned because it became clear that the technique was connected with many more difficulties than first imagined.

In Denmark, experiments in this field were initiated in 1958 when the first ^{60}Co plant was established in this country, and they increased with the building of the first linear accelerator here in 1960. To begin with several smaller and larger experiments were performed with a variety of food products. Later, the experiments were limited to sprout and growth inhibition in vegetables, because this was one of the areas where optimism lasted under our conditions, although not without difficulties and drawbacks.

The work presented here was performed during the years 1960-1976 at the Agricultural Research Department of the then Danish Atomic Energy Commission Research Establishment Risø, Roskilde, and it deals with a number of problems connected with the use of ionizing radiation for sprout and growth inhibition in vegetables.

Some of the results have been published earlier, but these papers will also be discussed in the present context in order to round off the whole work. The papers in question are given in chronological order below, only omitting a few preliminary or short notes that appear in the references.

I. Skou, J.P. (1963). Changes in the permeability of carrot tissues due to γ-irradiation and other physical and chemical treatments. - Physiol. Plant. 16, 423-441.

II. Skou, J.P. & J.B. Henriksen (1964). Increased susceptibility to storage rot in potatoes and carrots after sprout-inhibiting gamma-radiation. - Radiation Preserv. Second Scan. Meet. Stockholm, September 9-11, 1963. - IVA Medd. 138, 48-54.

III. Skou, J.P. (1964a). *Aureobasidium pullulans* (de By.) Arnaud. - A common and very radio-resistant fungus on fresh fruits and vegetables. - Ibid. 138, 63-70.

IV. Skou, J.P. (1964b). Radiation induced damage to plant tissues as a cause of the intensified attacks by microorganisms after irradiation. - Ibid. 138, 72-78.

V. Skou, J.P. (1966). On radiation-induced sprout inhibition and the by-effects. - Kerntechnik, Isotoptech. - Chem. 8, 510-513.

VI. Skou, J.P. (1967). Consumer surveys in Greenland for acceptance evaluation during storage of potatoes sprout-inhibited with γ-rays or Fusarex. - Risø Report No. 163, 30 pp.

VII. Skou, J.P. (1969). Notes on hyperplasia induced in carrots by β-rays, decapitation or *Agrobacterium tumefaciens*. - Radiation Botany 9, 397-406.

VIII. Skou, J.P. (1971a). Effects of calcium on storage rot in irradiated and non-irradiated carrots. - Acta Horticulturae 20, 115-124.

IX. Skou, J.P. (1971b). Studies on the effects of ionizing radiation for extending the storage lives of onions. - Risø Report No. 238, 46 pp.

X. Skou, J.P., K. Bech & Kirsten Lundsten (1974). Effects of ionizing irradiation on mushrooms as influenced by physiological and environmental conditions. - Radiation Botany 14, 287-299.

XI. Skou, J.P. (1978). On the intensified attack by microorganisms following irradiation-induced sprout-inhibition in vegetables. - In: The Use of Ionizing Radiation in Agriculture. Proceedings of a Workshop held at Wageningen, March 22-24, 1976. Commission of the European Communities: Biological Science EUR 5815 en, 131-156.

ABSTRACT

Several series of experiments have shown ionizing irradiation to be an effective method for sprout and growth inhibition in vegetables, but also that the doses should be kept at the absolute minimum necessary for the purpose to avoid unwanted by-effects. These doses are for potatoes and onions in deepest dormancy 8-10 krads and 6 krads, respectively; for carrots, which have no real dormancy, 12 krads are needed to reduce top sprouting satisfactorily, and for mushrooms 200 krads are needed for a week's prolongation of the shelf-life without considerable pileus expansion and stipe elongation.

Irradiation of the top end of carrots leaving the rest of the roots protected gives a good sprout inhibition and reduces the increase in susceptibility to rot, but it also results in an uncontrolled, secondary tumourous growth at the protected end of the carrots when st ed in a humid atmosphere. A comparable effect can be brought about by cutting the top end with a knife.

Potatoes, onions, and carrots suffer increased susceptibility to storage rot after irradiation with sprout-inhibiting doses. This is connected with the wounding and bruising caused by digging up and handling, as the wound-healing process is inhibited simultaneously with the sprout inhibition. In potatoes, a pre- and post-irradiation wound-healing period may minimize the problem because of their pronounced ability to form wound periderm, and also because suberization of exposed cells in the wounds may take place after irradiation and give some protection. Such a wound-healing period cannot be used in the case of carrots partly because their wound-healing capacity is too small, or develops too slowly, and partly because the surface of carrots dries out easily and loses turgescence, which results in an increased susceptibility to rot.

The basis for the increased susceptibility to rot after the application of sprout-inhibiting doses and the possibility to compensate for the irradiation-induced damages to the tissue was closer examinated with carrots as measuring object because of their pronounced susceptibility to rot after irradiation.

The pathogens increase tissue permeability in advance of growth during pathogenesis and as irradiation has an analogous

effect on tissue, it might facilitate the growth of the pathogens.

Irradiation increases the mobility of calcium in the tissue, possibly primarily that bound in the pectic substances of the middle lamellae, and may thereby make the tissue more accessible to microbial attack. At the same time irradiation has a softening effect on the tissue.

An external supply of calcium ($CaCl_2$) increases the firmness of tissue, reduces tissue permeability, and may compensate for the loss of calcium in irradiated tissue mainly as a result of a surplus of calcium in the wounds. Thus calcium has a considerable protective effect in the storage of wounded carrots, whether these are irradiated or not.

Botrytis cinerea and *Sclerotinia sclerotiorum* were some of the most widely spread and serious pathogens in the irradiated carrots studied. Attacks by *B. cinerea* increase upon drying the surface of the carrots, whereas attacks caused by *S. sclerotiorum* decrease. Culture filtrates from these organisms had a strong macerating activity on carrot tissue. The difference in this effect between culture filtrates of the two organisms was found to result from a thermostable factor produced in greater amounts by *S. sclerotiorum*. This factor exhibited an abrupt macerating or softening effect that stopped after about an hour's treatment, as it is the case when carrot tissue is treated with oxalic acid.

Calcium strongly reduced or prevented the macerating effect, and it completely nullified the effect of the thermostable factor.

Closer examinations showed that *B. cinerea* produces pectinesterase, polygalacturonase that is more active on pectic acid than on pectin, lyase that is very active on pectin, but weakly active on pectic acid, and oxalic acid at a slow rate. *S. sclerotiorum* produces pectinesterase, polygalacturonase equally active on pectin and pectic acid, none or extremely little lyase, and oxalic acid at a fast rate. Two other carrot pathogens, *Chalaropsis thielavioides* and *Mycocentrospora acerina*, produced small amounts of pectolytic enzymes only detectable through accumulation of their reaction products in the growth medium.

The activity of the single enzyme was examined under simul-

taneous action of the others as close to the situation in nature as possible. Under these conditions calcium enhanced the activity of pectinesterase, but the activity ceased at a lower level of total hydrolysis than it did in the presence of sodium ions or without salts. The activity of polygalacturonase was strongly reduced by calcium, whereas the lyase activity was weakly enhanced at low calcium levels and weakly reduced by an increasing concentration of calcium in the reaction mixture. The oxalic acid reduced the effect of calcium in proportion to the concentration and had obviously no effect on the enzyme activity proper.

Oxalic acid may act synergistically with the pectolytic enzymes in softening or macerating the tissue by removing calcium from the pectins of the middle lamellae.

The pectolytic enzymes mentioned are widely distributed among organisms and not confined to plant pathogens. Some pathogens even show very little or no pectolytic activity. Because of this, and because there exist pectolytic enzymes for every condition and pectic substance and because calcium is not very inhibitory to all kinds of pectolytic enzymes, it is not to be expected that the very obvious protective effect of calcium will always be expressed to the same extent on storage, as it will depend on the conditions and on which organisms constitute the main part of the pathogens. At the same time, the protective effect of calcium is reduced proportional to a reduction of wounding, for which reason the main part of the effect is suggested to be the result of an accumulation or surplus of calcium in the wounds. Thus, $CaCl_2$ is unusable as a protective measure against storage rot in general.

Another drawback to the use of sprout- and growth-inhibitory doses of irradiation is the internal discoloration of potatoes, onions and mushrooms. This effect, however, may be minimized by good growing and storage conditions, and for mushrooms, primarily by a packing technique that ensures good air exchange without a drying out of the product.

Sprout- and growth-inhibiting doses of irradiation hardly change the taste and texture of the products apart from the induced, transitory, sweet taste in potatoes.

By refutation of the drawbacks and by use of first-class products only, irradiation may be used to inhibit sprouting in potatoes, onions, and carrots and to prevent further growth in mushrooms.

1. INTRODUCTION

The sprout-inhibitory effect of ionizing radiation was first described as a usable practical measure in potatoes (Sparrow & Christensen 1954) and onions (Dallyn, Sawyer & Sparrow 1955) in the U.S.A., and shortly after in carrots (Mikaelsen, Brenna & Roer 1956) in Norway, while irradiation was first used for growth inhibition in mushrooms in The Netherlands several years later (Staden 1963). Since then very many papers have been written on the subject throughout the world. A larger number of them are referred to by Skou (1967 VI, 1971b IX, 1974 X, 1978 XI).

In Denmark interest in this technique appeared together with the general interest in and optimism concerning studies on the peaceful use of radiation, which reached a climax at the end of the fifties and in the early sixties. Interest was further promoted because of the disadvantages of the use of chemicals and other methods for sprout inhibition in storage (cf. e.g. Hansen 1953, 1955, 1961, Truelsen 1959a, Henriksen 1960, Reeve, Forrester & Hendel 1963a, b, Skou 1967 VI, 1971b IX, Augustinussen, Jørgensen & Huld 1975). This led to series of experiments with irradiation-induced sprout and growth inhibition in vegetables from 1958 (Truelsen 1959a, b) and onwards. The experiments with the various products were initiated at the request of governmental and private institutions (Government Crop Husbandry Research Service and Horticultural Department of the Royal Veterinary and Agricultural University, Copenhagen; Danish Onion Growers' Union; cf. Anonymous 1961, Skou 1971b IX).

Besides experiments with sprout and growth inhibition proper, the majority of the investigations during the following years were performed in order to solve problems that might prohibit the use of the measure in common practice. Several papers dealing with the details of these problems were published as mentioned in the preface and further information is given below together with a survey of the basis for the sprout- and growth-inhibitory effect of irradiation. The experiments include studies on the possibility of the versatile effects of calcium as a protective measure active on irradiated and non-irradiated plant tissues as well as on the pectolytic enzymes of the pathogens.

2. IRRADIATION-INDUCED SPROUT AND GROWTH INHIBITION IN VEGETABLES AND THE BY-EFFECTS

2.1. On the Inhibitory Effect of Irradiation

The voluminous literature dealing with the final causes of the observed inhibitory effects is not a matter for detailed discussion in connection with the present work, but a short outline of the complex nature of the effect will be given below.

The effects of irradiation are unspecific in the sense that all substances are affected where and when the radiant energy is absorbed. What we observe is only the differences in the resistance to irradiation of molecules, complexes of molecules, tissues or whole organisms, whether expressed physically, chemically, biochemically, physiologically, genetically or as inhibition, inactivation, death rate, etc. Kuzin (1964) set up excellent schemes in order to express the very complex nature of the processes leading from the radiation dose applied to the various effects observed.

These effects - lethal or non-lethal for the organisms - were studied after chronic or acute exposure to ionizing radiation. A most comprehensive review by Gunckel & Sparrow (1961) on many of the effects concluded that in too many cases the highly significant effects of irradiation on the growth and development of plants were not yet adequately tied to known changes at the cellular or biochemical levels. To the present author's knowledge this is still the case. Further, he is of the opinion that most of the morphological irregularities described are probably secondary effects due to physiological imbalance caused by genetic, cytological and physiological factors (cf. also Gunckel 1957).

Depending on dose and dose rate (acute or chronic irradiation), the morphological changes cover the whole range from growth stimulation (cf. e.g. Norreel & Rao 1974) over a variety of abnormalities on different parts of the plants, including fasciations and tumours, to growth inhibition or inactivation and plant death.

The morphological effects on plants following chronic exposure to ionizing radiation was most extensively studied in the γ-fields at Brookhaven National Laboratory (cf. e.g. Gunckel

et al. 1953; Gunckel & Sparrow 1954; Gunckel 1957, 1965; Gunckel & Sparrow 1961; Woodwell 1962, 1963; Sparrow 1966). These authors' results are in analogy with those of the present author. The suppression of lateral buds and overgrowth of inflorescences by clusters of modified leaves resulting in the production of a large number of vegetative shoots when the plants (*Ipomoea paludosa* E. Anders. & R.E. Woodson) were removed from the x-field (Gunckel et al. 1953) seem to be sprout inhibition and growth disturbance similar to the present author's observations regarding carrots (Skou 1969 VII). Comparable phenomena occurred on *Ipomoea fuba* (Schlecht.) G. Don, where leaves sometimes arose from the tumours indicating a partial recovery after chronic exposure to radiation at the Enivetoc nuclear test site (Biddulph & Biddulph 1953). The same applies to the fasciated modifications of axillary buds (*I. paludosa*) and the development of fascicles of adventitious roots on the stem of *Xanthium* sp. (Gunckel 1957).

The results of the present author (Skou 1969 VII) with carrots, which must be due to a secondary effect of irradiation, showed three kinds of overdevelopment or tumorous growth of the tissue: (1) An increase of the volume of the root end tissue with localized thickening of the root end as the only abnormal sign, (2) A callus-like outgrowth of the root traces or at the root end, and (3) at the same site tumours consisting partly of callus tissue and partly of clustered root initials imbedded in the callus tissue. Non-irradiated carrots developed small tumours in only 4 out of 7,545 fleshy roots. This is 0.05%. Carrots irradiated with 12-13.5 krads β-rays on the upper two cm of the top end developed from one to several larger or smaller tumours in 28-44% of the roots, depending on storage conditions and whether the roots were placed horizontally or vertically, normal or inverted. When an equally high dose of γ-rays was applied to the whole length of the carrots they never developed tumours (Skou 1969 VII). By comparing these results with those from chronic irradiation, and with others which appeared after acute irradiation (e.g. Bankes & Sparrow 1969), or after acute localized irradiation with heavy ionizing particles (Hirono, Smith & Lyman 1968), the question arises whether the tissue expansion, overgrowth, fasciations, and tumours are only different expressions of one and the same process, the intensity of

which depends on the dose level of irradiation and on the tissue conditions. Further, these abnormalities are affected by the growth conditions and by different secondary effects of irradiation such as changes in hormone content (Gunckel & Sparrow 1961), local injuries, and superabundant nutrition (White 1948, Gunckel & Sparrow 1954), but other by-effects should not be left out of account (cf. e.g. Skou 1966 V).

Already Linnaeus (1751) held the opinion that fasciation results from an increase in the number of growing points at such close positions that they subsequently fuse, but histological investigations have later indicated that fasciations are initiated from a single growth point (cf. White 1948). However, it need not be a question of either/or, as cutting off the top end of a carrot or injuring the growing root tip may wake or give rise to many growth points, which may develop into more or less clustered adventitious shoots or fasciations (cf. White 1948, Skou 1969 VII). This is in agreement with the present author's observations as summarized above, and it confirms that Linnaeus' opinion is just as good. Further, White concluded that the basic cause of fasciation is a disturbed metabolism and an unbalanced control of growth, which may finally result in abnormalities that have much in common with plant galls and cancer. This is in complete agreement with the results of partial irradiation of carrots (Skou 1969 VII).

Radiation-induced abnormalities in growing plants are one thing, but a quite different thing is the radiation-induced prevention or inhibition of sprouts and growth. The latter generally requires higher doses, and the total dose to prevent growth is much higher for chronic than for acute irradiation. A direct comparison between chronic and acute irradiation is irrelevant, however, because chronic irradiation results in a struggle to overcome radiation-induced damages, whereas acute irradiation results in a more or less complete and immediate cessation of any growth activity - possibly apart from some "remnant activity". On the other hand, plants that are relatively resistant to acute exposure to irradiation are also relatively resistant to chronic exposure and vice versa (Sparrow 1966).

There are considerable differences in the resistance of different species (Sparrow & Christensen 1953; Sparrow 1966),

as well as within the single species due to the variety and the physiological condition. This point will be discussed below for some vegetable products.

2.2. Author's Results and Discussion

The material and methods for each series of experiments in this paragraph has been presented in detail for each product in the previous papers listed in the preface, for which reason they are not included here. Results from series of experiments with carrots are presented below, whereas the results with potatoes (Skou & Henriksen 1964 II, Skou 1966 V, 1967 VI, 1970), onions (Skou 1971b IX, Skou & Iversen 1972), and mushrooms (Skou, Bech & Lundsten 1974 X) only will be discussed briefly.

2.2.1. Carrots (*Daucus carota* L.)

Sparrow (1966) compared the interphase volume of the nucleus of plant cells with the irradiation doses necessary to produce severe growth inhibition (defined as 15% of control) in a large number of plant species and found an inverse proportionality. The predicted resistance on this basis is extracted from Sparrow (1966) for carrots and for the extreme cases (Table 1).

These results should be compared with the general resistance outlined in Figure 1.

The results with carrots were in good agreement with the radiation resistance of callus cultures of carrot tissue (Norreel & Rao 1974) and with the 12 krads found necessary for sprout inhibition by the present author (Skou 1960, 1966 V, 1978 XI), but this dose is much lower than the 50 krads that Holsten, Sugii & Steward (1965) call a non-lethal dose for cell and tissue cultures of carrot.

Results of irradiation-induced sprout inhibition in carrots for short-time storage were given in the papers of Skou (1966 V, 1978 XI), in which the effect of partial irradiation is also discussed. The results of long-term storage of irradiated carrots are given below.

2.2.1.1. Sprouting. In the 1963/64 experiments with 12 krads it was noted that considerable sprouting occurred on the non-irradiated carrots on removal after half a year's storage at

Table 1. Predicted resistance of carrot tissue on exposure to acute or chronic irradiation compared to the most susceptible and the most resistant plants found. The 95% confidence limits are in brackets. (Extracted from Sparrow 1966 Table 6)

Plant	Interphase volume μ^3±SE	Irradiation	
		acute, r	chronic, r/20-hr day
Tulipa fosteriana Hoog, HV Red Emperor	86.2±6.8	401 (283-568)	36 (28-47)
Daucus carota L.,cv.	4.0±0.2	8,586 (6,061-12,162)	777 (601-1,005)
Sedum alfredii Hance var. *nagasakianum* Hara	0.7±0.02	50,899 (35,931-72,101)	4,608 (3,563-5,960)

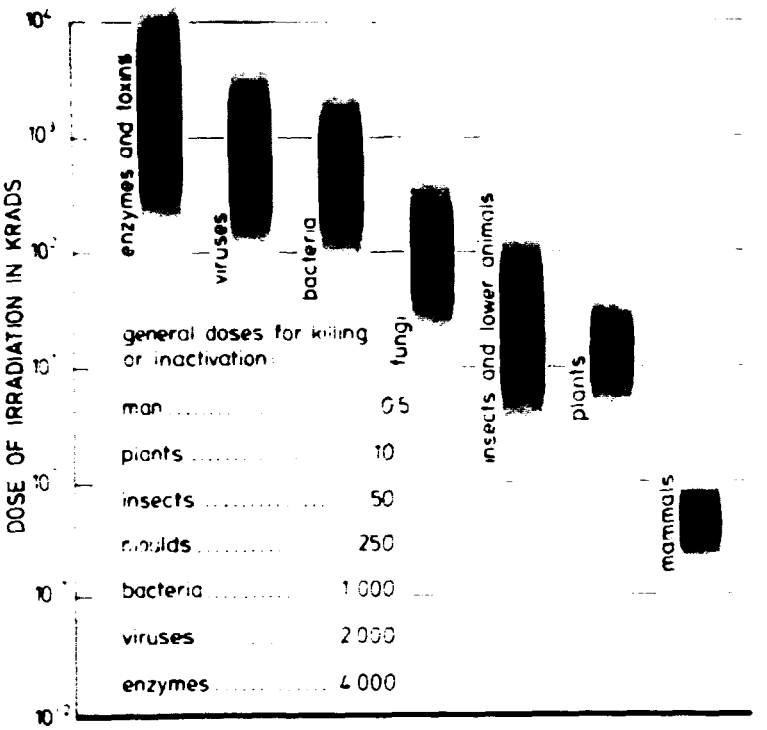

Figure 1. Outline of the radiation resistance of different groups of organisms, and of matters important to human foodstuffs. The blacker the column, the more members of the group in question have their limit for resistance on the corresponding level of irradiation. The figure gives an impression of why it is difficult to use irradiation for the preservation of fruit and vegetables. For comparison, the thickness of the baseline gives the dose (10 rads) permitted by the health authorities without a special licence (Anonymous 1967).

0-2°C and close to 100% r.h., whereas only traces of sprouts were noticed on irradiated carrots. The development of sprouts after removal was not noted.

The non-irradiated carrots from the 1964/65 experiments had a high percentage of sprouts on top and root on removal from storage. Irradiation with 12 krads reduced the number of carrots with top sprouts to 6% or less of those in the non-irradiated material and prevented development of roots at the

root traces (Table 2). Measurements of the fresh weight of the top sprouts after 14 days at room temperature gave an even better illustration of the difference in sprouting, as it was hardly possible to weigh the small amounts of sprouts on the irradiated carrots (Table 3). The lower number of sprouted carrots and the lower sprout weight per carrot in the control may be due to drier conditions, because these carrots were not protected by perforated plastic bags. The wound-healing period and the $CaCl_2$ treatment had little effect on the sprouting.

Also in the 1965/66 experiments the unprotected control carrots showed reduced sprouting - mainly of the roots - compared to those packed in perforated plastic bags (Table 4). This must have something to do with the relative humidity although it was kept close to 100%. In this year a greater number of carrots sprouted after irradiation than in the preceding years' experiments, but again the root sprouting was prevented. When the carrots in these experiments were kept at 10°C and at room temperature, nearly 100% of the non-irradiated carrots sprouted (Table 5). There is no definite explanation why carrots irradiated in January and March had a relatively high percentage with top sprouts on removal from storage but it could be connected with a higher turgescence caused by the washing at that later time. The difference disappeared on the further storage (Table 5) and it was not expressed in the weight of the sprouts (Table 6).

The difference between sprouting at 10°C and at room temperature, and the relatively large amounts of sprouts in the irradiated carrots, is also clearly expressed by the weight of the top sprouts (Table 6). While the effect of temperature may be explained as primarily an effect of the lower relative humidity, the greater amounts of sprouts in the irradiated carrots in the 1965/66 experiments than in those of the preceding years may or may not be based on differing resistance of the 'Hafnia' and 'Touchon' varieties used, or on the variation between the years.

The roots developed to a very large extent in all non-irradiated carrots, except in those of the drier untreated control, and the extent was greater at 10°C than at room temperature, which supports the above assertion. In the irradiated carrots there was only sporadic root development (Table 6).

Table 2. Percentage of carrots with top sprouts and fine, white, hairy roots at the root traces on removal after 5.5 months' storage at 0-2°C and a r.h. of close to 100%, 1964/65 experiments. Variety 'Hafnia'.

Treatment	Washed		Washed + 0.02N $CaCl_2$		Washed + 0.04N $CaCl_2$	
	top	root	top	root	top	root
Control, not washed	(61.2)	(14.9)				
Non-irradiated						
No wound-healing period*)	83.9	63.9	80.3	84.0	76.1	71.4
Wound-healing period	83.5	82.5	79.8	81.9		
Double wound-healing period			78.7	76.3		
Irradiated with 12 krads						
No wound-healing period	5.0	0	4.4	3		
Wound-healing period after irradiation	2.5	0	3	3	0.1	0.4
Wound-healing period before irradiation	2.0	0	3.2	0	0	3
Double wound-healing period before irradiation			3.7	0		

*) Here a wound-healing period means that the carrots were kept for 7 days at 7°C and 70% r.h. but it is questionable if any wound-healing process occurred. However, possible wounds dried up during the period.

Table 3. Average weight in grammes of top sprouts per carrot 14 days after removal from storage and keeping at room temperature. 1964/65 experiments. The same carrots as those referred to in Table 2.

Treatment	Washed	Washed + 0.02N $CaCl_2$	Washed + 0.04N $CaCl_2$
Control, unwashed	(0.56)		
Non-irradiated			
No wound-healing period	0.84	0.77	0.79
Wound-healing period	0.66	0.82	
Double wound-healing period		0.97	
Irradiated with 12 krads			
No wound-healing period	traces	traces	
Wound-healing period after irradiation	0.004	traces	0.15
Wound-healing period before irradiation	traces	traces	traces
Double wound-healing period before irradiation		traces	

Table 4. Percentage of the carrots with top sprouts and fine, white, hairy roots at the root traces on removal from storage at 0-2°C and a r.h. of close to 100%. 1965/66 experiments. Variety 'Touchon'. Irradiation dose 12 krads.

Storage period after the treatment	Untreated control*)		Non-irradiated				Irradiated			
			Washed		Washed + $CaCl_2$**)		Washed		Washed + $CaCl_2$**)	
	top	root	top	root	top	root	top	root	top	root
Nov.-Jan.	1.5	0	5.8	0	15.9	0	0	0	0	0
Nov.-March	5.9	0.3	71.2	7.5	79.9	9.9	1.2	0	0.6	0
Nov.-May	22.9	0.4	87.2	47.2	83.7	38.0	1.8	0.1	2.3	0
Jan.-March	7.9	0.3	82.3	9.5	84.2	10.8	17.0	0	10.3	0
Jan.-May	21.3	0.1	87.5	36.8	86.3	39.4	3.7	0	7.6	0
March-May	16.4	0.6	86.7	29.8	84.4	27.1	5.5	0	5.4	0

*) Stored unprotected. Other carrots were proteoted by perforated plastic bags (6-10 carrots in each) (cf. Skou 1969).

**) 0.2% $CaCl_2$, $2H_2O$ ∿ 0.027N $CaCl_2$ for 2 hours after the washing.

Table 5. Percentage of the carrots that developed top sprouts and fine, white, hairy roots at the root traces when kept at 10°C or at room temperature (half the replicates at each) after removal from storage. 1965/66 experiments. The same carrots as those referred to in Tables 4 and 6.

Storage period after the treatment	Number of days after removal	Untreated control				Non-irradiated								Irradiated							
						Washed				Washed + $CaCl_2$				Washed				Washed + $CaCl_2$			
		10°		room temp.		10°		room temp.		10°		room temp.		10°		room temp.		10°		room temp.	
		top	root	top	root	top	root	top	root	top	root	top	root	top	root	top	root	top	root	top	root
Nov.-Jan.	18							99.0	65.2			97.3	75.4			46.5	0.2			80.8	1.8
Nov.-March	15/33*)	94.3	59.9	98.9	8.0	91.2	85.2	98.7	87.6	97.4	96.3	96.1	86.7	56.9	0.5	64.7	1.1	55.2	0	73.7	1
Nov.-May	15	96.3	43.1	68.8	0	98.6	95.7	98.9	89.7	99.3	96.5	97.5	88.0	68.5	0.4	54.8	2.2	77.4	2.4	86.4	1
Jan.-March	15/33*)	93.8	46.6	96.9	0	89.1	99.3	97.8	82.2	99.7	100	94.8	79.9	46.5	0	47.4	0	66.2	1.2	64.4	0.7
Jan.-May	15	90.7	25.3	67.3	0	97.1	89.3	96.7	81.7	100	100	99.1	89.3	43.7	0	41.4	0	64.6	1.6	51.7	0.4
March-May	15	77.9	20.2	74.3	4.4	100	94.3	96.9	92.7	99.0	96.9	99.2	81.2	30.7	0	10.1	0	30.3	0	20.2	0

*) 15 days at room temperature, 33 days at 10°C.

Table 6. Average weight in grammes of top sprouts per carrot after removal from storage and keeping at 10°C or room temperature. 1965/66 experiments. The same carrots as those referred to in Tables 4 and 5.

Storage period after the treatment	Number of days after removal	Untreated control		Non-irradiated				Irradiated			
				Washed		Washed + $CaCl_2$		Washed		Washed + $CaCl_2$	
		10°	room temp.	10°	room temp.	10°	room temp.	10°	room temp.	10°	room temp.
Nov.-Jan.	-	-	-	-	-	-	-	-	-	-	-
Nov.-Marcn	15/33*)	0.64	0.37	1.16	0.63	1.48	0.80	0.33	0.25	0.36	0.34
Nov.-May	15	0.54	0.24	1.13	0.62	1.11	0.59	0.35	0.18	0.47	0.29
Jan.-March	15/33*)	0.52	0.16	1.30	0.58	1.47	0.41	0.13	0.04	0.48	0.42
Jan.-May	15	0.45	0.21	1.06	0.42	1.15	0.60	0.10	0.03	0.34	0.24
March-May	15	0.34	0.20	0.92	0.59	1.02	0.68	0.05	0.04	0.04	0.03

*) 15 days at room temperature, 33 days at 10°C.

The development of sprouts after commercial treatment gave the same overall picture as described above, but the severe rotting in the irradiated carrots as described below continued destructively although all rotten specimens were discarded on removal from storage (Table 7, and cf. Skou 1978 XI).

2.2.1.2. Rotting. In connection with irradiation-induced sprout inhibition in carrots, intensified rotting is the paramount problem - as discussed in several papers (cf. Skou 1978 XI and Tables 8-12). Some of the present author's results with short- and long-term storage were previously reported (Skou 1960, 1971a VIII, 1978 XI, Skou & Henriksen 1964 II). These and other results are discussed below.

There are two fundamental causes of the intensified rot: the relative susceptibility of plant tissue to irradiation and the much higher resistance of microorganisms (Figure 1). This would not constitute any problem if irradiation could be used for sterilization of fresh plant tissue, but this cannot be done because high doses change the plant tissue so that it loses its attractiveness for human consumption, and also because of the fear that the formation of unknown, unwanted substances may increase with increasing doses. Therefore the dose should be kept as low as possible.

Preliminary experiments with carrots showed that not only was the growth of pathogens intensified after irradiation, but also that such organisms as *Penicillium* sp., *Mucor* sp. and *Aureobasidium pullulans* (de By.) Arn. (syn. *Pullularia pullulans* (de By.) Berkh.), which did not grow on non-irradiated carrot disks, grew well after irradiation of the disks with high doses. Further, *A. pullulans* exhibited increased growth on carrots treated with increased dose of irradiation from 250 to at least 1,000 krads when inoculated on surface-sterilized carrots, whereas organisms such as *Stemphylium* sp. and *Fusarium* sp. did not grow after doses above 250 krads (Skou 1960). A closer examination of *A. pullulans* showed it to be one of the most radio-resistant fungi ever found (Skou 1964 III, Kovaltsova, Zakharov & Levitin 1970, Partsch & Altmann 1972).

On malt extract agar, *Botrytis cinerea* Pers. ex Fr. was found to be one of the most resistant microorganisms, requiring a "lethal" dose of above 470 krads (Skou 1960). A radio-resist-

Table 7. Commercially treated carrots. Percentage of the carrots with top sprouts and fine, white, hairy roots at the root traces on removal from storage at 0-2°C and a r.h. of close to 100% - and the development of top sprcuts and the amount of fresh carrots after keeping at 10°C or room temperature for 17 days. For the amount of rot on removal from storage, see Skou (1978 XI).

	Non-irradiated				Irradiated			
	Washed		Washed + $CaCl_2$		Washed		Washed + $CaCl_2$	
	top	root	top	root	top	root	top	root
Sprouts and roots on removal	15.8	4.9	23.1	7.2	0	0	0.2	0
Sprouts and roots at 10°C 17 days after removal	83.1	33.5	86.6	45.9	2.3	0	4.4	0
Sprouts and roots at room temp. 17 days after removal	59.6	5.1	61.8	10.9	0.9	0	2.3	0
g sprouts/carrot after 17 days at 10°C	0.46		0.47		0.002		0.005	
g sprouts/carrot after 17 days at room temp.	0.18		0.20		0.001		0.025	
% fresh carrots after 17 days at 10°C		48.9		61.1		1.1		13.0
% fresh carrots after 17 days at room temp.		63.8		72.2		8.2		24.9

ance on the same level for this fungus and other important plant pathogenic fungi is reported by Beraha, Smith & Wright (1960) and Beraha (1964).

For studying the growth of microorganisms on irradiated and non-irradiated carrots, slices of carrot were placed in sterile water in petri dishes in order to keep them turgescent. All the organisms tested grew well in this water when the slices were irradiated, but not when non-irradiated. This led to the suggestion of a radiation-induced increase in the permeability of the carrot tissue (Skou 1960).

In the following years the effect of irradiation on carrot tissues was investigated in connection with the use of calcium in order to see if this cation could compensate for the induced changes in permeability and thereby increase the resistance against microbial attacks (Skou 1963 I, 1964b IV, 1971a VIII).

Calcium was chosen on the basis of the following facts, partly in order to throw more light on the cause of the effect, partly in order to see if it could be used as a protective measure in common practice:

1. Irradiation increases the tissue permeability (cf. Skou 1963 I, 1964b IV).
2. Pathogens increase the tissue permeability (cf. e.g. Thatcher 1939, 1942, Lai, Weinhold & Hancock 1968, Wheeler & Hanchey 1968).
3. Ca^{++}-ions decrease the tissue permeability (cf. Skou 1963 I, 1964b IV) and give the plant material a harder texture (cf. Kertesz 1951 and others, see below).
4. Ca^{++}-ions affect the activity of pectolytic enzymes produced by the pathogens (cf. the investigations below and the literature review in the appendix).

An interaction between these four parameters might have a positive effect on carrot tissue, which could perhaps make it possible to overcome the increased susceptibility to microbial attacks after irradiation.

The first experiments with a $CaCl_2$-treatment did not significantly reduce the intensity of rot in the irradiated carrots (Skou & Henriksen 1964 II). However, they were perhaps not given the best possible handling, as how to give the best con-

ditions for wound-healing were unknown.

The results of the 1963/64 experiments showed a pronounced difference in rot between non-irradiated and irradiated carrots. In the non-irradiated carrots there was an insignificant tendency to reduced rot in those treated with $CaCl_2$ and to a negative effect of the wound-healing period. In the irradiated carrots the $CaCl_2$-effect was in all cases significant, whereas the effect of the woundhealing period in general was clearly negative (Table 8).

The pattern of effect in the 1964/65 experiments was in general the same as that of the year before. Irradiation seriously enhanced the rotting, the double wound-healing period had a clearly negative effect, and the protective effect of the $CaCl_2$-treatment was significant in both non-irradiated and in irradiated carrots (Table 9).

In experiments in the following years (1965/66) the wound-healing period was omitted and the carrots were handled as gently as possible (cf. Skou 1971a VIII). The amount of rotting was small compared to that in the preceding years' experiments. These two facts contribute to the relatively small negative effect of irradiation. These facts reduced the protective effect of $CaCl_2$ to a tendency (Table 10). The amount of rot increased with the storage period, but appeared to be independent of the delay between harvest and irradiation.

After removal of the rotted carrots, half the replicates were placed at $10^{\circ}C$ and the other half at room temperature. Under these conditions the further development of rot was very heterogeneous, but the negative effect of irradiation was still provable (Table 11).

In the same year (1965/66) an experiment set up with commercial treatment of the carrots (Figure 2) resulted partly in a 120-140% increase in rot after irradiation and partly in a 30-40% decrease in rot as a result of the $CaCl_2$-treatment (Skou 1971a VIII, 1978 XI).

A similar protective effect of calcium referred in the literature for other crops or vegetable products is discussed in chapter 8.

Looking over the years of experiments, the general impression is that the gentler the handling, the less rot and the less effect of the treatments. Regardless of irradiation, wash-

Table 8. Per cent rotted carrots in storage from November 1963 to May 1964.

Treatment	Not washed	Washed	Washed + 0.02N $CaCl_2$	Significance between untreated and $CaCl_2$-treated
Non-irradiated				
No wound-healing period	3.2 ± 0.9	2.7 ± 0.6	1.4 ± 0.2	
Wound-healing period *)	2.8 ± 0.9	4.3 ± 1.0	2.1 ± 0.8	
Irradiated with 12 krads				
No wound-healing period	17.5 ± 4.6		10.1 ± 1.5	P < 0.001
Wound-healing period after irradiation	31.8 ± 4.2	32.6 ± 5.0	18.5 ± 2.7	P < 0.001
Wound-healing period before irradiation	33.0 ± 2.5	22.3 ± 2.4	7.2 ± 1.2	P < 0.001

*) Wound-healing period in this case means keeping the carrots for 3 days at 6-8°C and a r.h. of 50-60%, but it is questionable if any wound-healing process occurred. Compare the 1964/65 experiments, Table 2.

Table 9. Per cent rotted carrots in 5.5 months' storage (Nov.-May) 1964/65.

Treatment	Washed	Washed + 0.02N $CaCl_2$	Washed + 0.04N $CaCl_2$	Significance of $CaCl_2$	
				0.02N	0.04N
Control, unwashed	(8.6 ± 1.8)				
Non-irradiated					
No wound-healing period	8.9 ± 2.5	5.1 ± 0.9	3.4 ± 1.3	P < 0.05	P < 0.05
Wound-healing period *)	6.7 ± 1.2	2.5 ± 1.1		P < 0.05	
Double wound-healing period		12.5 ± 2.5			
Irradiated with 12 krads					
No wound-healing period	37.2 ± 3.5	(48.5 ± 3.4)			
Wound-healing period after irradiation	38.0 ± 2.6	37.8 ± 2.3	25.0 ± 3.0	no difference	P < 0.05
Wound-healing period before irradiation	33.0 ± 5.0	33.3 ± 4.0	26.1 ± 4.6	no difference	P < 0.05
Double wound-healing period before irradiation		46.8 ± 2.8			

*) See the footnote to Table 2.

Table 10. Per cent rotted carrots in the 1965/66 experiments on removal from storage.

Storage period after the treatment	Untreated control	Non-irradiated		Irradiated		
		Washed	Washed + $CaCl_2$*)	Washed	Washed + $CaCl_2$	
Nov.-Jan.	0.4 ± 0.2	1.2 ± 0.5	0.5 ± 0.2	4.2 ± 1.3	3.2 ± 0.9	
Nov.-March	4.1 ± 1.8	5.3 ± 1.8	3.1 ± 0.7	11.0 ± 2.1	9.4 ± 1.7	
Nov.-May	3.2 ± 1.4	3.1 ± 1.2	4.2 ± 1.2	12.2 ± 2.5	21.6 ± 5.0	**)
Jan.-March	2.0 ± 0.9	3.5 ± 1.7	2.6 ± 0.9	9.1 ± 2.2	8.5 ± 1.4	
Jan.-May	7.2 ± 4.1	4.4 ± 1.3	9.8 ± 1.5	13.2 ± 2.5	14.8 ± 1.9	
March-May	2.8 ± 1.1	0.5 ± 0.4	1.5 ± 0.6	7.2 ± 1.6	6.6 ± 2.1	

*) 0.027N $CaCl_2$ = 0.2% $CaCl_2$, $2H_2O$.

**) In two of the six replicates there was an exceptional amount of rot.

Table 11. Development of the rot (in per cent) when the carrots were kept at 10°C or at room temperature (half the replicates at each temperature) after removal from storage. 1965/66 experiments.

Storage period after the treatment	Number of days after removal	Untreated control		Non-irradiated				Irradiated			
				Washed		Washed + $CaCl_2$ *)		Washed		Washed + $CaCl_2$ *)	
		10°	room temp.	10°	room temp.	10°	room temp.	10°	room temp.	10°	room temp.
Nov.-Jan.	18				2.6		6.5		17.0		11.3
Nov.-March	15/33**)	26.7	1.1	18.6	2.8	6.2	8.7	19.3	26.7	19.9	15.0
Nov.-May	15	1.2	1.6	0.3	3.1	1.8	2.5	3.9	13.4	5.4	14.7
Jan.-March	15/33**)	3.9	3.1	16.2	7.0	3.8	13.3	26.9	28.1	28.4	27.1
Jan.-May	15	12.0	13.4	7.0	1.6	2.2	0.9	4.0	6.6	10.8	2.1
March-May	15	29.8	11.5	0.3	1.5	3.5	2.3	9.2	10.9	10.5	16.1

*) 0.027N $CaCl_2$ = 0.2% $CaCl_2, 2H_2O$.

**) 15 days at room temperature, 33 days at 10°C.

Figure 2. Machinery for washing carrots. Note the rotating washing-drums and the conveyers to the table for sorting and packing.

ing may or may not cause intensified rotting (cf. also Årsvoll 1969 and Jørgensen & Jensen 1975).

In the 1964/65 experiments observations were made on the organisms causing the rot. Roughly speaking, they show the same pattern as the percentages of rot (compare Tables 10 and 11). *Botrytis cinerea* and *Sclerotinia sclerotiorum* (Lib.) de By. were the organisms most often present as "pure" infections, but there was a difference in their occurrence. The number of attacks by *B. cinerea* increased when the carrots were given a wound-healing period, whereas attacks by *S. sclerotiorum* decreased (Table 12). This observation is in close agreement with the results of Mukula (1957), who prevented infection by *S. sclerotiorum* by washing and drying, by disinfection with flame or with boiling water, whereas such treatments increased the decay from *B. cinerea* attacks. This is reasonable because the fungus might have been present as an incipient infection (cf. Goodliffe & Heale 1975) and then, through treatment, have obtained better conditions for development. These conditions are obviously brought about by the drying-out of tissues (wilting) and by an increase in the osmotic potential, as Djacenko (1971) found decreasing attacks by *S. sclerotiorum* and increasing at-

Table 12. Number of carrots attacked by different microorganisms on removal from storage. About 510 specimens observed per treatment. The 1964/65 experiments.

Treatment	Washed				Washed + 0,02N $CaCl_2$				Washed + 0,04N $CaCl_2$			
	B. cinerea	*S. sclerotiorum*	rot*)	total	*B. cinerea*	*S. sclerotiorum*	rot*)	total	*B. cinerea*	*S. sclerotiorum*	rot*)	total
Control, unwashed	(1)			(1)								
Non-irradiated												
No wound-healing period		4	2	6	1	2	18	21	3	2	15	20
Wound-healing period	2	6	1	9	1	1	3	5				
Double wound-healing period					13	3	19	41				
Irradiated with 12 krads												
No wound-healing period	25	23	17	65	43	17	12	61				
Wound-healing period after irradiation	44	11	41	85	26	4	2	32	16	4	13	33
Wound-healing period before irradiation	37	3	14	54	43	3	14	41	13	2	12	27
Double wound-healing period before irradiation					44	4	22	70				

*) Attacks by, *Alternaria*, *Fusaria*, bacteria, or other organisms, more or less in mixture and therefore not easily identified. Mixed infections now and then involving *B. cinerea* and *S. sclerotiorum* included.

tacks by B. cinerea under increasing dryness and osmotic potential, which is in agreement with Årsvoll (1969), who stated that B. cinerea never attacks carrots kept in good physiological conditions, but loss of turgidity, mechanical injuries or attack by other pathogens facilitate attack by B. cinerea. Very recently the observations with B. cinerea were further confirmed by Goodliffe & Heale (1977).

These observations inspired the study of the effects of the two fungi in closer detail as described in the remainder of this paper.

The aim of giving the carrots a wound-healing period was to heal any wounds originating from lifting and handling, analogous to the effect in potatoes, or at least to dry the surface and wounds of the carrots, but it was unknown if the process was positive or effective enough. The results proved that it was not, as a few days at 50-70% r.h. in general resulted in increased rotting. This is in agreement with the facts discussed above and with those of Lentz (1966), Apeland & Baugerød (1971), and Djacenko (1971), who found considerable drying out at all temperatures and at r.h.'s below 98.5%, and that this was connected with increased rot. These results agree with the results and observations after long-term storage as discussed above. In agreement with this, the results of van den Berg & Lentz (1966, 1968) and van den Berg & Yang (1969) clearly showed that 90-95% r.h. during long-term storage gave more rot than 98-100% r.h. On the basis of the present author's results given above, however, it is supposed that the effect of drying out of the carrot surface was already achieved after a few days in storage; though Lauritzen (1932) found an increased rot with increasing r.h.

The reason may lie in the fact that the wound periderm hardly develops readily enough to make a considerable protective barrier and also that carrots only have a scarcely developed periderm at lifting (Mukula 1957). Further, this is why carrots easily lose and regain water during storage (Lentz 1966, Djacenko 1971).

With reference to the effect of irradiation, it is assumed that periderm formation is inhibited, in analogy with the case in potatoes. This will be discussed together with the effect of calcium.

2.2.2. Potatoes (*Solanum tuberosum* L.)

Chronic irradiation of potato plants with 80 r (X-rays) per day and a total of 8.5 Kr had no measurable effects on growth and yield (Sparrow & Christensen 1950), whereas 300 and 600 r per day for ten weeks had a mild or severe growth-inhibitory effect, respectively (Sparrow & Christensen 1953, Gunckel & Sparrow 1954). Acute exposure of the potatoes to ionizing radiation below 300 r had no significant effect on growth and yield, whereas a dose of 4.8 Kr reduced the yield to 4% of the control, and thus was close to the lethal limit for irradiation of 'Katahdin' potatoes (Sparrow & Christensen 1950). Later these authors stated (Sparrow & Christensen 1954) that 20 Kr would prevent sprouting and improve the storage quality. Doses of this magnitude, however, may give rise to increased susceptibility to microbial attack in the potatoes, for which reason doses of 8-12 krads are nowadays used or recommended for this purpose (cf. Desrosier & Rosenstock 1960; Skou 1978 XI).

Germination of potatoes was strongly inhibited or prevented with 8.0 Kr when applied in the dormant state, whereas this dose gave 25% sprout development on an average if applied after initiation of germination (Heiken 1960). Jaarma (1960), who used 15 krads, also found increasing resistance to irradiation with increasing delay between harvest and irradiation.

When the potatoes were planted in the field an increasing delay of emergence with increasing dose of radiation was observed and a strong decrease in yield resulted after all doses (0.5-8.0 Kr) applied, and it augmented with the dose (Heiken 1960).

Differences have been observed between potato varieties regarding radiation resistance, the variety 'Bintje' being one of the more sensitive (Heiken 1960, Jaarma 1960; cf. also Skou 1978 XI).

Apart from some preliminary experiments with sprout inhibition (Truelsen 1959a, b, 1960) only 8 krad doses were applied in our investigations as the primary aim was to inhibit sprouting and to maintain storage quality. In all these experiments 'Bintje' was used and only sporadic sprouting occurred after irradiation - even under the most extreme conditions (Skou 1967 VI).

Wound periderm formation is a type of growth which is at least as susceptible to irradiation as sprouting. The resulting loss in wound-healing capacity is a serious drawback to the method, because it causes easier access for microbial attack (cf. Henriksen 1960, Skou & Henriksen 1964 II, Skou 1967 VI, 1978 XI), though this may be overcome by giving the potatoes a wound-healing period before irradiation. This should be compared with the effect of sprout-inhibiting chemicals, which exhibit an analogous effect on wound-healing (cf. e.g. Cunningham 1953, Reeve, Forrester & Hendel 1963a, b and the discussion by Skou 1978 XI).

The suberization that occurs in the existing cells in the exposed wound tissue is more resistant to irradiation and may even be produced in more layers in the irradiated wound than in the non-irradiated (Skou & Henriksen 1964 II, Olafsson 1976). The suberization of the wound tissue may have a protective effect against storage rot (Smith & Smart 1959) and may play an important role in connection with the good results sometimes obtained by giving the potatoes a wound-healing period after irradiation (cf. Skou 1978 XI).

The use of first-class potatoes in good physiological condition makes it possible to maintain the quality through irradiation as the increase in rot will be of little significance compared to the sprouting and increased weight loss in non-irradiated or Fusarex treated potatoes. If potatoes of poorer quality are used, irradiation further reduces the quality (Skou & Henriksen 1964 II, Skou 1967 VI, 1970, 1978 XI).

2.2.3. Onions (*Allium cepa* L. and others)

Doses as low as about 100 r affect the transpiration of onions (Srb & Petránková 1966), and in the range of 100 to 1,000 r there is an increasing effect on cell permeability (Hluchovský & Srb 1963, Srb & Hluchovský 1963, Srb 1964, 1965).

Chronic gamma irradiation with 400 r or 800 r caused mild or severe effects, respectively, on the growth of onions (Sparrow & Christensen 1953). The sprout inhibitory effects of acute irradiation are very dependent on the physiological conditions of the onions. Doses in the range of 2-5 krads applied when the onions are in deep dormancy may prevent sprouting (cf. the re-

view of Skou 1971b IX).

One of the drawbacks to the use of irradiation-induced sprout inhibition in onions is the discoloration of the sprout bud or internal sprouts, though this does not occur in cold storage and only after several months' storage. It has something to do with the radiation dose, the physiology of the onions, and the cultural variety (cf. e.g. Skou 1971b IX, Temkin-Gorodeiski, Kahan & Padova 1972), but there is no definite explanation. It seems most likely to implicate growth physiology and the dying-out of the growing centre.

As in the case of potatoes, an immediate and a delayed increase in rot may occur after irradiation (Skou 1978 XI), and these may have their causal basis in wounds and bruises, or in physiological changes, respectively, though the types of tissue are very different from those of potatoes.

2.2.4. Mushrooms (*Agaricus bisporus* (Lange) Singer)

Fungi are in general much more resistant to irradiation than are higher plants. The resistance of fungi depends on the species in question and on the growth medium; the resistance of plant pathogens is higher in plant tissue than on artificial media (cf. e.g. Beraha, Smith & Wright 1960, Săvulescu 1966, Skou 1964a III, 1978 XI). These facts contribute to make irradiation-induced sprout inhibition a difficult task because these dose levels inactivate or kill the plant cells, but leave the pathogens almost unaltered and with easier access to the plant tissues as discussed above (cf. Figure 1).

As mentioned above, doses high enough to inhibit or kill the fungi cannot be used for raw vegetables because they will cause a pronounced softening (cf. e.g. Massey 1968). At a dose level of 200-300 krads the shelf life of soft fruits may be prolonged without considerable softening, but the growth of micro-organisms is only delayed.

Data on irradiation of agarics is so limited that nothing can be said in general, but the radiation resistance of mushrooms is on the same level as that of fungi on the whole, though it depends on the parameter used for the determinations. Increase in diameter of pilei or in length of stipes during storage involves changes in shape and volume, and may or may not be re-

garded as real growth. It cannot be brought to a complete stop by 300 krads. The same holds for opening the veils that is a ripening process hardly involving any growth. Mushroom mycelium growing out of pilei and stipes as a mould fluff during storage may be significantly inhibited with 50 krads and is extinct after 200 krads (Skou, Bech & Lundsten 1974 X).

Microbial attacks on mushrooms that are growth-inhibited with ionizing radiation seem no problem. There is, however, internal discoloration, though this is very dependent on the physiological condition of the mushrooms and on the packing material and method (Skou, Bech & Lundsten 1974 X). This drawback is obviously not a question of respiration as this decreases with increasing doses (Kovács & Vas 1974), rather it may be caused by enzyme activity in connection with increased permeability of the cells (cf. Bacq & Herve 1952, Yamaguchi & Campbell 1973). The skin colour of the irradiated mushrooms during storage is generally better than that of the non-irradiated mushrooms if not packed too tightly (Kovács & Vas 1974, Skou, Bech & Lundsten 1974 X).

2.3. Commercialization

The significance of several parameters must be estimated for every single product before this new method can be released for use in common practice. The more important of these parameters are taste, texture, discoloration, wholesomeness, and economic considerations, but there are several others (Skou 1966 V, 1978 XI).

The irradiation of potatoes results in some release of sugar from starch, which gives a sweet taste that lasts until the sugar is metabolized, and some lots of potatoes have a tendency to internal blackening that increases on irradiation (Truelsen 1964, Skou 1966 V, 1967 VI, 1970, 1978 XI). On an international scale all these problems are regarded as small, and wholesomeness studies have revealed no adverse effects from feeding on irradiated potatoes. For this reason the health authorities in several countries - including Denmark - have permitted the use of sprout-inhibiting doses of irradiation of potatoes for human consumption (on application according to Government Notice (Anonymous 1967)).

Economically, however, an irradiation plant cannot be based on potatoes alone because it must necessarily be in function during the whole year and because this product cannot bear expensive transport costs, for which reason the plant would only receive potatoes from its immediate surroundings (Skou 1970).

Sprout-inhibiting doses to carrots gave no change in taste and texture, insofar as a panel of 17 of author's colleagues were unable to differentiate between carrots treated with 0, 12, 18 and 25 krads. If there are any differences, they can only be tested by a panel of experts. Analyses of the caroten content after the same doses revealed no significant differences (analysed by Statens Vitamin-Laboratorium). Neither was it expected because of the high resistance of caroten to irradiation (Lukton & MacKinney 1956, Franceschini et al. 1959).

Few wholesomeness studies have been made on irradiated carrots, and no country has permitted the use of the method on carrots for human consumption. In principle, the economic considerations may be the same as for potatoes, but the possible irradiation of this product is not limited to a specific season.

As in the case of carrots, the irradiation of onions with sprout-inhibiting doses gave no changes in taste and texture, but the sprout bud or internal sprouts may discolorate, as mentioned above. The health authorities of several countries have released this product for human consumption. Regarding the economic considerations, it should be taken into account that the irradiation of onions is limited to a rather short season of the year (Skou 1971b IX, Skou & Iversen 1972).

The use of growth-inhibitory doses of irradiation on mushrooms only had adverse effects on the flesh colour, and this was strongly dependent on the physiology of the mushrooms and on the packing method (Skou, Bech & Lundsten 1974 X). Nevertheless, up to now only the health authorities of the Netherlands have released this product for human consumption (Anonymous 1969). Economically, mushrooms are a good subject for irradiation as there can be a continuous flow of products for irradiation by the plant the year round, and production can be concentrated in the neighbourhood of the plant.

Finally, but not least, there is the serious, emotional, and psycological barrier to overcome before irradiated products can win common public acceptance. This may be illustrated by

the Dutch mushroom project, which has been reputed as a gigantic flop (Friedman 1977). On the market, the mushrooms were labelled "irradiated" or "fresh", respectively, and why buy irradiated mushrooms when one can get them fresh?

2.4. Summary

The effects of irradiation are unspecific and may be expressed in different ways as resistance of substances, cells, tissues or organisms.

Depending on dose and dose rate, and on whether the irradiation is acute or chronic, the effects of irradiation span the whole range from growth stimulation over various abnormalities and growth inhibition to plant death.

The radiation-induced development of abnormalities seems to be a result of more or less local injuries that suppress or prevent normal growth and budding. Depending on the conditions of the remaining, undisturbed living tissue, this starts uncontrolled adventitious growth, which may develop into tumour-like structures that are more or less imbedded in callus. The plants try to overcome the damage by compensating growth, which depends on the severity of the damage and the regenerative ability. This shows an inherent tendency in plant tissues that all that can grow will grow, and no matter the cause of damage the growth will develop more or less uncontrolled. The present author's results with carrots are an example of this feature.

Evidence that the described effect is not caused by a special property of the β- and γ-rays was found in the fact that cutting the top end with a knife also resulted in the development of tumours. Further, the positioning of the carrots greatly influenced the size and the number of tumours, which implies that hormones may play a part in the results. Concerning the fasciculate origin of the tumours, it is characteristic of the tumours, or the hyperplasias, in carrots that they are initiated de novo at many points with irregular whorls of tracheary elements, as observed in histological examinations (Skou 1969 VII).

In carrots with the top end cut, or irradiated with β-rays, the fine hairy, white roots at the root traces develop undisturbed as in the case of healthy untreated carrots. Doses higher than 4-5 krads will prevent growth of these roots. Complete pre-

vention of sprouting at the top end is rather difficult; though 12 krads strongly retards the growth, there is still some growth after 25 krads (Skou 1966 V, 1978 XI).

Temperature and relative humidity interact in their effect on sprouting. There are indications that carrots irradiated in November sprout more than carrots irradiated in January. These carrots again sprout more than those irradiated in March. This is the opposite of the case with potatoes and onions and may be connected with differences in physiology, as carrots have no distinct period of dormancy. The amount of sprouting in the irradiated carrots differs from one year to another, but it is not clear if this is a difference between the varieties used or a variation between years. It is clear, however, in all cases that 12 krads are most suitable for practical purposes.

In the literature there are reports on differences between potato varieties regarding their resistance to sprout inhibitory doses of irradiation; 'Bintje' is one of the most susceptible varieties. The experiments show that only sporadic sprouting occurs in this variety after irradiation with 8 krads, even when it is stored under the most extreme conditions (Skou & Henriksen 1964 II, Skou 1967 VI).

There are some indications that sprouting in potatoes is most susceptible to irradiation in the dormant state (Heiken 1960, Jaarma 1960). For sprout inhibition in onions it is a decisive requirement for a good result that they are irradiated in deep dormancy in the first few weeks after harvest. At that time 6 krads strongly reduce the sprouting ability, whereas 12 and 25 krads give a higher percentage of sprouted onions. This effect is regarded as a kind of dormancy break. Irradiation at a later time may only inhibit sprouting with difficulty (Skou 1971b IX).

As irradiation inhibits sprouting, it also inhibits the formation of a new periderm, whereas the suberization of existing cells may take place. This leads to a loss of wound-healing capacity and an easier access for microbial attack (Skou & Henriksen 1964 II). A good pre- and post-irradiation wound-healing period may almost eliminate this drawback in potatoes, but only first-class products should be used because use of second-class potatoes with more or less latent infections may result in an increased rate of deterioration (Skou & Henriksen 1964 II, Skou

1967 VI, 1970, 1978 XI).

Carrots have less wound-healing capacity than have potatoes, consequently it is doubtful whether a wound-healing period in carrots would be of any protective value. At least this treatment cannot be used in the same way as with potatoes, because a relative humidity of below 98-100% results in water loss and altered turgidity, which rapidly increase the accessibility to microbial attack. Irradiation greatly increases the susceptibility to rot in carrots. Even after extremely gentle handling in order to avoid wounding, there is considerably more storage rot in irradiated carrots than in untreated carrots. Any commercial handling would result in so much rot in the irradiated carrots that it would be prohibitive to use the method for long-term storage, unless the irradiation was limited to the top end of the carrots, or they could be protected against rot in another way. For short-term storage, that means for about a month, at the greengrocer's, or for long transport after removal from the storage room (0-2°C), irradiation may be a practical measure for sprout inhibition in carrots.

Botrytis cinerea and *Sclerotinia sclerotiorum* are among the most frequent pathogens on carrots in storage, but their frequency alters because they behave differently according to the effect of the wound-healing conditions used.

Also onions may suffer increased storage rot after irradiation, but careful handling may keep it at a relatively low level for half a year (Skou 1971b IX).

These problems do not exist in connection with irradiation of mushrooms with growth-inhibiting doses of 200 krads, which is sufficiently inhibitory to control both mould fluff and pathogens such as, e.g., *Mycogone perniciosa* (Magn.) Del. and *Pseudomonas tolaasii* Paine (Skou, Bech & Lundsten 1974 X).

In irradiated potatoes, onions and mushrooms, internal discoloration may be a problem after irradiation. In potatoes it is obviously an increase of an already existing tendency that may be further enhanced by growing the potatoes under unbalanced conditions. In onions the discoloration is exclusively limited to the sprout bud or internal sprouts. It seems to be connected with the physiology and cultural variety of the onions, so irradiation only enhances an already existing tendency. It does not occur in cold storage. Also in mushrooms the tendency

to internal discoloration depends on the physiological conditions, but also to a large extent on the packing material and method.

Apart from the fact that potatoes get a sweet taste, which under good storage conditions is transitory, sprout and growth inhibitory irradiation does not affect the taste and texture of these products.

In Denmark, the health authorities have approved the irradiation of potatoes for human consumption, as is the case in several other countries. In some countries, irradiated onions are also approved, while only The Netherlands permit irradiated mushrooms too.

3. REVIEW OF THE EFFECT OF IRRADIATION ON VEGETABLE STORAGE TISSUES

Already Brasch & Huber (1947) noted texture changes in different vegetables after irradiation, but they did not point out any specific dose. Later, Hannan (1955) found that 2 Mrep gave an appreciable loss of texture in vegetables, e.g., in carrots, by using a rough, subjective scoring method (0-xxx, compare Brown's method (1915)) for detection of enzymatic maceration of plant tissues. In the following experiments the subjective factor was avoided by the use of different instruments for measuring the texture of vegetables.

McArdle & Nehemias (1956) thus estimated the texture of carrots from tenderometer readings on 100 g of 0.25 inch diced cubes of the roots and found an almost linear decrease in texture from about 225 lb/sq.inch to about 60 lb/sq.inch in the range of 0 to 2.5 Mrep, giving a CD_{50} (50% reduction in crushing load) dose for decrease in firmness of 1.6 Mrep (calculated by the present author). It is not reasonable to expect the linear relationship to continue down to the very small doses, but if it did, the decrease in firmness due to the application of sprout-inhibiting doses should not be more than half a per cent, as calculated from the results of McArdle & Nehemias (1956).

Glegg et al. (1956) measured the crushing loads of carrot cylinders, 18 mm in diam. and 10 mm long, by pressing a piston against them with a constant actuating hydraulic force. By this method they found crushing loads for carrots of 90-92 1.3-2.3 lb and a threshold dose of 166 Kr. Further, the firmness decreased proportionally to the logarithm of the dose. Boyle et al. (1957) and Kertesz (1957) used the method of Glegg et al. (1956) for examining the crushing loads and CD_{50} values for 7 lots (5 varieties) of carrots. The extremes found for the threshold doses for crushing loads were 23.5 krads and 178 krads, and the CD_{50} was 671 krads and 2,310 krads, respectively.

The threshold doses and the CD_{50} values seem independent factors which vary considerably both within and between varieties. Thus the threshold doses were 23.5 krads and 137 krads and the CD_{50} values 1,860 and 774 krads, respectively, for two lots of 'Long Imperator' carrots. The lot with the lowest threshold dose had the highest crushing load for non-irradiated spec-

imens, 124±3.1 lb, while 'Chantenay' with the highest threshold dose had the lowest initial crushing load, 92±1.2 lb.

Comparing the crushing load for non-irradiated specimens in all lots with the corresponding threshold doses shows that in 5 out of 7 lots the higher the crushing load (initial firmness), the lower the threshold dose. Boyle et al. (1957) and Kertesz (1957) mention that changes in turgor pressure might influence radiation-induced softening, but the authors only do this speculatively and without referring to any facts in their own results.

Massey (1968) used a modified Instron Universal Testing Machine (a commercial machine that measures the compressive and tensile properties of foods by transferring the force used to a strip-chart recorder that draws a force-distance curve, cf. Bourne, Moyer & Hand 1966) in which standard-sized pieces of carrot tissue were pressed between two parallel plates. Massey was thus able to measure properties of the tissue such as hardness, elasticity, cohesiveness, and gumminess that is a force function defined as the product of hardness and cohesiveness. The results of the hardness studies were a threshold dose of 30 krads at the 10% confidence level followed by a decrease in hardness that was proportional to the logarithm of the dose. Also the elasticity of the carrot tissues decreased with increasing doses of irradiation. For this parameter the author calculated a threshold dose of 80 krads. Based on these experiments, Massey (1966, 1968) postulated that the initial effect of radiation on the texture of raw carrots is that of destruction of the selective permeable characteristics of the cell membranes for water (compare Skou 1963 I).

Irradiation-induced softening was also studied in several other products. In a comparison of heating and ionizing radiation of potatoes, Roberts & Proctor (1955) found that 70°C disturbed the pectic substances of the middle lamellae and, though it did not destroy the cellulose, it weakened it so much that the cells were no longer able to retain the starch grains. In comparison, 2-3 Mrep altered the middle lamellae but not to the breaking point, so that the cells were still able to retain starch grains after irradiation. As was the case with carrots, McArdle & Nehemias (1956) found a linear decrease of firmness in apples with increasing irradiation and without any threshold

dose, but the apples appeared much softer than the carrots. Glegg et al. (1956) found that apples had a lower threshold dose than had beets and carrots, but the slopes of the curves for the decrease in firmness were almost parallel for the three products, which means that the degradation rate was equal. The results of Boyle et al. (1957) and Kertesz (1957) were in agreement with those of Glegg but they showed no relationship between the crushing load of the non-irradiated apples and the threshold dose as it appeared from their figures with carrots. Kertesz (1957) found no significant decrease in firmness in potatoes after applying 270 krep, but after 542 krep and higher doses the decrease was significant. Salunkhe (1958/59) noted that softening was directly correlated with the irradiation dose in Jerusalem artichokes (*Scorzonera hispanica* L.), but he only found a significant decrease after applying more than 2 Mrep, and he does not mention anything about the testing method he used. Later, he (Salunkhe 1961) scanned the literature for the effect of irradiation on several vegetables including carrots. On this basis, he concluded that 200-300 krads caused no inacceptable softening from the point of view of consumption.

All detailed quantitative investigations agree that there is a threshold dose for the irradiation-induced softening of vegetables, but that this dose varies considerably between species and varieties and within the single variety. It is a question whether the differences in turgor and in the age or quality of the products are more important for the threshold dose and crushing load than are the varieties. This point should be compared to the problems with carrots discussed below.

The smallest threshold doses mentioned in the literature lie close to the highest doses necessary for sprout inhibition. These facts raise the question of whether there could be a connection between the decrease in firmness - degradation of the tissue - and the intensified attack by microorganisms after application of sprout-inhibiting doses of ionizing radiation.

The next question is how closely related is irradiation-induced softening to the degradation of the cell wall constituents, such as cellulose and pectin? According to the target theory of direct action on large molecules and the kinetics of indirect actions of radicals in aqueous solutions, the dose of irradiation required to produce chemical changes is inversely

proportional to the molecular weight (Lea 1955). Therefore, it is to be expected that large molecules, such as cellulose and pectin, may undergo some depolymerization even at the sprout-inhibiting dose level.

Saeman, Millett & Lawton (1952) showed that cellulose depolymerizes at high-dose levels, being significant between 10^5 and 10^6 equivalent roentgens. The depolymerization is random along the molecular chain with an increased production, with increasing doses, of easily hydrolyzable material and sugars. The presence of lignin obviously gave some protection.

Later, Glegg & Kertesz (1956) and Glegg (1957) found that cellulose is significantly degraded by about 100 Kr with some further degradation as an after-effect when stored in the dry state. The effect was measured by viscosimetry with the cellulose solution in cupriethylenediamine. The primary effect of irradiation was proportional to the logarithm of the dose. Kertesz (1957) and Kertesz et al. (1964) found a threshold dose of 28.4-81.2 krep γ-rays for degradation of cellulose from carrots and beets and compared the results with the threshold doses for crushing load and the corresponding slope of the curves for the irradiation-induced softening. Based on this, they concluded that the doses for degradation of cellulose are equal to or less than the doses required for softening the plant tissues. The presence of water had no significant effect on the extent of the irradiation-induced degradation, but with a very low moisture level an after-effect was measured, as mentioned above.

To this it should be added that irradiation with γ-rays rendered cellulose more susceptible to enzymatic degradation (Okada, Kraunz & Gassner 1960).

McArdle & Nehemias (1956) found linearity between decrease in total pectic substances and the irradiation dose in the range of 0-2 Mrep for pectic substances from both apples and carrots. Primarily, the protopectin degraded to pectin and pectates, the content of which increased with increasing doses. However, this was not the only effect as the viscosity of all three fractions decreased with increasing doses of irradiation, indicating that the molecules undergo depolymerization.

Kertesz et al. (1956) and Kertesz (1957) irradiated both dry pectin and 0.5% solutions of pectin and showed in both cases the degradation to be proportional to the logarithm of the doses

when measured as a decrease in viscosity. The pectin was very stable when irradiated in the dry state, but, as in the case of cellulose, an after-effect was observed that was most pronounced in the first ten days after irradiation (Herz & Kertesz 1956, Kertesz 1957). With the 0.5% solutions it was found that both the viscosity and the Ca-pectate-value of the pectin decreased significantly at a dose level of 8.3 Kr, and a threshold dose for irradiation-induced degradation of pectin was calculated to 3.8 Kr (Kertesz et al. 1956). Pectin solutions were strongly protected against degradation by several sugars and tartaric acid due to their jellying effect. At a low pH with a high sugar concentration, protection was absolute within the range of doses used (0-212 Kr). These facts indicate that pectin may be much more stable in situ than in vitro but, in contrast to the case of cellulose, the water content played an important role for the degradation of pectin. Kertesz (1960) investigated the effect of water content on the resistance of pectin to 340 krads and found a very steep increasing degradation of the pectin with increasing dilution starting with a moisture content of about 75-80% (Figure 3).

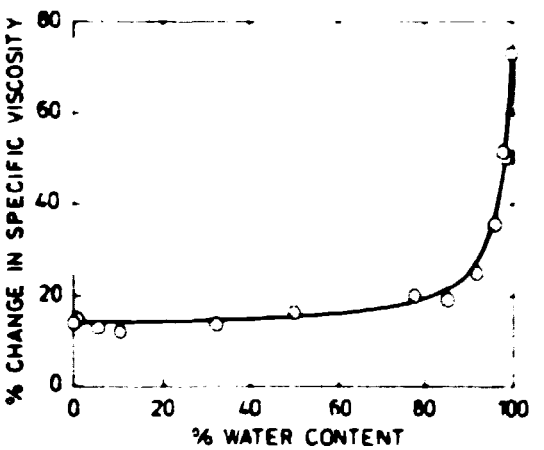

Figure 3. The influence of water content on the radiation resistance of pectin measured as per cent change in specific viscosity. Irradiation dose 340 krads. (Drawn after Kertesz 1960 Table 8).

Skinner & Kertesz (1960) found through electrophoretic studies that irradiation breaks the glucosidic linkages of the pectin at random along the molecular chain by hydrolytic fissure. This results in a lower molecular weight and explains the decrease in viscosity with increasing irradiation, though the effect was little up to 205 krads and only after 528 and 2,056 was it pronounced. In agreement with these findings, Džamić & Janković (1966) calculated on the basis of viscosimetric investigations a drop in molecular weight from 115,000 in a non-irradiated 0.1% solution of pectin to 2,000 after the application of 2 Mrads. Their results also show a pronounced drop in viscosity already after 20 krads. At the same time the esterification decreased from 41.9% in non-irradiated pectin over 39.7% after 20 krads to 21.9% after 2 Mrads.

In close agreement with others (Kertesz et al. 1956, Kertesz 1957), Deshpande (1965) demonstrated the degradation of pectin in a 0.5% solution down to 4 krads measured as a decrease in viscosity and in molecular weight. Further, he showed that the degradations of pectin by irradiation and by enzyme treatment (pectinesterase or pectinase) were not additive processes when the pectin was pretreated with the enzymes. On the other hand, the enzymatic degradation of pectin was enhanced if it was applied as a post-irradiation treatment, which may be connected with a decarboxylation caused by the irradiation (Shah 1966) and the resulting release of calcium.

Later it was found that the susceptibility of a 1% pectin solution to pectinesterase (PE) increased with doses up to 250 krads and decreased for polygalacturonase (PG) and pectate lyase (Romani, Somogyi & Manalo 1971). At least the higher PE activity may influence the mobility of calcium after irradiation.

The present author finds these results very important in connection with studies on the causes why irradiated vegetables suffer increased susceptibility to microbial attacks. Therefore series of experiments were performed in order to see if an irradiation-induced softening of the tissues could be responsible for the easier access of microorganisms at the sprout-inhibitory level and if a calcium treatment could compensate for this effect.

3.1. Summary

The literature on the effect of irradiation on the texture of plant tissues has been reviewed. The smallest threshold dose for the softening effect referred to in the literature is close to the highest dose needed for sprout-inhibiting purposes. Cellulose is significantly degraded by 100 krads and higher doses, whereas the threshold dose for depolymerization of pectin was found to be as low as 3.8 krads.

It was further indicated that the degradation of pectin induced by irradiation was not additive to that caused by pectolytic enzymes; however, the enzymatic degradation was enhanced if applied as a post-irradiation treatment.

Irradiation caused a release of calcium. This may be an effect of the irradiation-induced depolymerization, demethylation and decarboxylation of the pectin, and it may possibly constitute the basis for the enhanced enzyme activity.

As the interaction between pectin and calcium is decisive for the firmness of plant tissue, it is clear that any disturbance of this is an important factor in the irradiation-induced softening of plant tissue.

4. EFFECT OF CALCIUM ON PLANT TISSUES AND MICROORGANISMS

4.1. Previous Investigations

4.1.1. Effect of Irradiation on the Mobility of Calcium

There are several reasons why plant products suffer intensified attack by microorganisms after irradiation (Skou 1960, 1964b IV, 1966 V, 1971b IX, 1978 XI, Skou & Henriksen 1964 II). Even doses at the level of 1 krad may cause immediate and short-term changes as well as irreversible damage to cell permeability (Hluchovský & Srb 1963, Srb & Hluchovský 1963, Srb 1964, 1965, Stadelman 1969). Higher doses of ionizing irradiation induce increased permeability of the tissues, cell death (Heilbrun & Mazia 1936, Higinbotham & Mika 1954, Skou 1963 I, 1964b IV, Massey 1966, 1967, 1968, Stadelman 1969), and degradation of pectic substances (Kertesz et al. 1956, McArdle & Nehemias 1956, Kertesz 1957, Deshpande 1965, Džamić & Janković 1966, Shah 1966), which are supposed to be some of the more important causal parameters as they are analogous to decisive properties of the pathogenic microorganisms themselves.

The release of electrolytes from carrot tissues increases exponentially with the irradiation dose (Skou 1963 I), and Ca^{++} was the cation that most readily - relative to the content - leached from the irradiated carrots (Echandi & Massey 1970). A radiation-induced calcium release was also found in other plant tissues using 200-600 krads, and in addition the irradiation of calcium pectate also resulted in a release of calcium (Al-Jasim & Markakis 1965, Al-Jasim, Markakis & Nicholas 1968). In this connection it should be mentioned that calcium may have a protective effect through the ionic bondings in the pectin molecules (Skinner & Kertesz 1960, Deshpande 1965). Together with the degradation of pectic substances, degradation of cellulose (Saeman, Millett & Lawton 1952, Glegg & Kertesz 1956, Glegg 1957), and liberation and release of Ca^{++}-ions from the irradiated tissue (Heilbrun & Mazia 1936, Al-Jasim & Markakis 1965, Al-Jasim, Markakis & Nicholas 1968, Massey 1966, 1967, 1968, Echandi & Massey 1970) contribute to the irradiation-induced softening of plant tissues, and hence possibly to easier access for microorganisms (Glegg, Boyle, Tuttle, Wilson & Kertesz 1956, McArdle & Nehemias 1956, Boyle et al. 1957, Kertesz 1957, 1960, Salunkhe

1958/59, Kertesz et al. 1964). Shah (1966) found the release of calcium from plant tissues proportional with the dose up to 600 krads, and in experiments with Ca-pectate or pectic acid in water suspensions he found a certain degree of decarboxylation, indicating that the electrovalent links between calcium and the pectic substances are broken on irradiation. On this basis Shah (1966) concluded that the release of calcium plays an important role in the radiation-induced softening of fruits and vegetables. Such an effect may be the reason for the decreased amounts of calcium pectate and increased amounts of soluble pectin found in strawberries after irradiation with 200 krads (Belli-Donini & Stornaiuolo 1969, Belli-Donini 1973).

The total calcium content of carrot cell walls amounts to approximately 45% of the total cellular calcium, and it is associated with the most insoluble polysaccharide fraction (Echandi, Chase & Massey 1970). In the present author's opinion the binding is rather to the pectines and pectinic acids that are made insoluble through binding with the calcium. Though calcium undergoes a redistribution due to solubilization by the irradiation, Echandi, Chase & Massey (1970) concluded that the release of calcium is either unassociated with the cell wall, or just a part of the weakly bound calcium and not responsible for the softening process. This is a rather different conclusion from that based on earlier experiments.

The effect of irradiation on the enzyme activity is discussed in the previous paragraph.

4.1.2. Effects of Calcium on Plant Tissues

The effects of calcium on plant tissues are multitudinous, physical as well as physiological and biochemical (Jones & Lunt 1967). In connection with the present work, mainly the parameters having relation to the above-mentioned effects and the effects of calcium during microbial attacks will be discussed below.

Calcium changes the permeability of living cells (Jennings 1963) and the physiological effects are best reflected by its action in decreasing cell permeability (Nason & McElroy 1963), which is observed in both non-irradiated and irradiated tissues (Skou 1963 I, 1964b IV). On the other hand, the loss of Ca^{++}-

ions, e.g. when exposed to EDTA (ethylenediaminetetraacetic acid) (van Steveninck 1965), increases the membrane permeability (Jones & Lunt 1967), which supports the supposition that the irradiation-induced release of Ca^{++}-ions has a similar effect. The effect of calcium on the membranes is most likely related to its binding to phospholipids (Jones & Lunt 1967); however, the binding of calcium to cuticular membranes (tomato fruits and onion leaves) is obviously greater on the inner than on the outer surfaces and the permeability for ions greater inwards than outwards (Yamada, Wittwer & Bukovac 1964, Yamada, Bukovac & Wittwer 1964). Also the cell walls exhibit an affinity for Ca^{++}-ions that is enhanced by the action of pectinesterase, which sets the carboxylic groups free (Somers 1973). In this connection it should be mentioned that the results of Jordon et al. (1973) indicated that calcium contributes to stabilizing the permeability of the plasmalemma of root cells to water.

The linkage between carboxylic groups of the molecular chains of pectic substances through the Ca-binding increases the rigidity of the cell walls (Jones & Lunt 1967), possibly by establishing a three-dimensional network (Isherwood 1955), whereas calcium is necessary for the cells proper to build up the elasticity of the cell walls (Bärström 1954).

Treatment of plant tissues with $CaCl_2$ after demethylation with pectinesterase would exercise a firming effect and make the pectic constituents of the tissue less accessible to microbial attack (Deshpande 1959, 1965, Brown 1969).

The practical use of the ability of calcium to increase the firmness of plant tissues was developed in 1937 (Loconti & Kertesz 1941, Kertesz 1951), and the method is now used in the food industry for several products including carrots (Anonymous 1968). Further, the use of calcium treatment for refirming irradiated plant products has given promising results (Markakis & Nicholas 1967, Al-Jasim, Markakis & Nicholas 1968).

Calcium-deficient plants were more susceptible to diseases than plants receiving normal amounts of this nutrient (Corden & Edington 1960, Deverall & Wood 1961a, Maynard et al. 1961, Corden 1965), and the pectic substances in calcium-deficient plant tissues were more easily hydrolyzed by pectolytic enzymes than those in normal tissue (Echandi, Corden & Dimond 1961). It should be mentioned in this connection that the oxalic acid

produced by some plant pathogens during pathogenesis is able to remove the calcium bound to pectic substances in the tissues and thereby to act synergistically with the polygalacturonases (Bateman & Beer 1965); further, ammonium oxalate is commonly used for extraction of pectic substances from plant tissues (cf. e.g. Simpson & Halliday 1941, Henglein 1955).

An external application of calcium salts, an increase of calcium nutrition, or a high level of calcium available in the soil may enhance resistance against diseases (Bloom & Walker 1955, Mohr 1955, Edington & Walker 1958, Mohr & Watkins 1959, Corden & Edington 1960, Deverall & Wood 1961a, Sitterly 1962), and the resistant plants or varieties contain more calcium than do the susceptible (Mohr 1955, Mohr & Watkins 1959, Thomas & Orellana 1964, Bateman & Lumsden 1965). Worley & Morton (1964), however, tried to confirm the positive results with southern blight of tomato caused by *Sclerotium rolfsii* Sacc. but without success. Another way of rendering the tissue more resistant was shown by Bateman (1964) and by Hancock & Stanghellini (1968), who found an accumulation of Ca^{++}-ions in the tissue adjacent to the lesions or in the lesions caused by the pathogen. There was no accumulation in the hyphae and the process seems specific for Ca^{++}-ions, as the amount of Mg^{++}-ions was about the same in healthy and in attacked regions (Hancock & Stanghellini 1968). In this context it is worth mentioning that Mohr & Watkins (1959) asserted that the reduced susceptibility of tomato plants to attack by *S. rolfsii* was due to calcium-induced increase in meristematic activity of the phellogen. This may increase the phelloderm formation and accelerate the wound-healing process.

The effects of calcium on non-irradiated and irradiated plant tissues and on plant resistance to diseases constitute the arguments for investigating the protective effect of calcium treatment of carrots for storage. These arguments are supported by the results of Mukula (1957), who found the total rot in carrots during storage reduced from 50% in untreated soil to 30.5% in soil to which was applied 5 tons/ha of CaO. The rot was almost exclusively caused by *Botrytis cinerea* and *Alternaria radicina* Meier, Drechs. & Eddy, and the effect was especially a reduction of the attack by the latter, which was reduced from 41.5% to 22.5%. Further support is found in Nikolaev &

Kholmkvist (1963) who stored the carrots in contact with chalk. Other investigations with application of calcium (Skou & Henriksen 1964 II, Skou 1971a VIII, 1978 XI, Jørgensen & Jensen 1975) also stimulated the studies of its causal effects.

4.1.3. Analyses of Calcium and Pectic Substances in Carrots

Disturbances of normal growth, or susceptibility to microbial attack due to lack of or deficiency in calcium, as discussed above and in an earlier paper (Skou 1971a VIII), are outside the scope of the present work.

There are, however, as mentioned in the preceding discussion, indications of increasing resistance against disease through the external application of calcium or a higher content of calcium available in the soil. The calcium content of carrots as cited in the literature is given in Table 13.

Table 13. The calcium content of carrots as cited in the literature.

Percentage of calcium in		Authors
fresh weight	dry matter	
	0.376	Robinson, Steinkoenig & Miller 1917
0.051	0.486	Mack & Herrmann 1934
0.022-0.044		Donelsen et al. 1943
	0.21-0.28	Maynard et al. 1961
	0.376-0.502	Altman & Dittmer 1964*
0.042-0.064		Schuphan 1965
	0.334-0.436	Medvedeva & Konovalova 1965

*Summary of several authors.

Donelsen et al. (1943) based their results on two varieties, one with 0.027-0.044%, the other with 0.022-0.031% Ca^{++} in the fresh weight, but the difference may hardly be regarded significant.

Maynard et al. (1961) obtained no increase in Ca^{++} content in the root dry matter by increasing the nutrition level from 20 ppm to a level of 400 ppm.

The calcium content of carrots seems to vary more from one year to another than from one region to another (Medvedeva & Konovalova 1965).

Miyamoto et al. (1957) found approximately 40 % of the calcium soluble and considered the insoluble calcium present as phosphate, carbonate, and oxalate tightly bound in the vegetable tissues. Nothing is mentioned about binding to pectic substances. These results are, however, not very much different from the results of Maynard & Gentile (1963), who found about half the calcium in carrot tissue in the soluble fraction, about 25 % bound to the cell walls, and 25 % in a particulate fraction.

Exactly how calcium is bound to the cell walls, or to the pectic substances in cell walls and middle lamellae, remains unknown as the results are very dependent on the extraction method used.

Elwell & Dehn (1939) extracted the pectic substances with 90°C hot water for one hour, Simpson & Halliday (1941) with boiling water for 25 minutes, and Kertesz et al. (1964) with water at 30°C. This makes a consistent difference between the fractions. Bennett (1944) removed sugar and pigments with ethanol and extracted the pulp with dibasic ammonium citrate. The amount of pectic substances is high, regarded as pectic acid, but small as total pectic substances (Table 14), though Henglein (1955) refers to a comparably small amount (0.62 % and 7.14 % based on wet and dry materials, respectively), as does Kertesz (1951) (6.9 % without mentioning the method used). Kertesz et al. (1964) extracted their samples with calgon (Na-metahexaphosphate) and NaCl before extraction with dilute acid, which may be the reason for the small fraction resulting from the last extraction (Table 14). Buston & Kirkpatrick (1931) found more than double as much pectic substance in the phloem parenchyma tissue (p.p., the tissue between the cambial region and the cork/periderm layers, most of which is parenchyma (Havis 1939, Esau 1940/41, Cutter 1971, Poole 1976)) as in the stele, but it is hardly credible that it should be possible to detect the amount of pectin in the middle lamellae just by subtraction of the amount soluble in dilute acid from the total amount.

Table 14. Pectic substances in carrots as cited in the literature.

Authors	Determined as	Pectin soluble in water %	Pectic substances soluble in diluted HCl or H_2SO_4 %	Pectic acid soluble in NH_4-oxalate or NH_4-citrate %	Calgon and NaCl extracted pectin %	Pectin in middle lamellae %	Total pectic substances %
Based on dry matter:							
Elwell & Dehn 1939	ethanol precipitate	7.12	11.45				18.57
	Ca-pectate	10.80	6.02				16.82
Simpson & Halliday 1941	Ca-pectate	3.7	14.1	0.8			18.6
Bennett 1944	Ca-pectate			7.41			7.41
Based on fresh weight:							
Buston & Kirkpatrick 1931	(det. in phloem parenchyma)		2.82			0.1	2.92
	(det. in stele)		1.17			0.1	1.27
Elwell & Dehn 1939	ethanol precipitate	0.97	0.54				1.51
	Ca-pectate	0.64	1.03				1.67
Kertesz et al. 1964	Ca-pectate	0.356	0.189		0.337		0.882

The best estimate of the amount of free carboxylic groups and the binding of calcium may be made from the results of Fertesz et al. (1964), who used the most gentle extraction method, though for the same reason they may not have extracted the total amount of pectic substances. They found about 38% of the pectic substances in the calgon-NaCl-fraction, which may be regarded as the part with more than 50% free carboxylic groups cross-linked with Ca^{++} (or Mg^{++}), and therefore insoluble in water (Kertesz 1951) but soluble in calgon that forms chelates with Ca^{++}. However, it cannot be disregarded that some Ca^{++} is also bound to the water soluble fraction with more than 50% esterified carboxylic groups and, at least partly, to cell-wall-bound pectic substances. The explanation of the difficulty in obtaining uniform results from extraction regarding the total amount, and to defining what are genuine pectic substances, may lie in the fact that the substances are more complex than earlier expected and are not confined to the middle lamellae but constitute a continuous matrix with the microfibrils of the primary and the secondary cell wall in gradually decreasing amounts towards the lumen of the cells (cf. e.g. Aspinall 1973, Keegstra et al. 1973, Bateman & Basham 1976).

4.2. Author's Analyses of Calcium in Carrots

4.2.1. Material and Methods

These analyses had four objectives: (1) to determine whether calcium moves through the cork/periderm layers into the depths of the phloem parenchyma (p.p.) tissue when the carrots are immersed in a $CaCl_2$ solution, (2) to determine whether the $CaCl_2$ film left on the surface after treatment could be of significance as a protective measure, (3) to determine the calcium content in different depths of the tissue, and (4) to see if there are differences between non-irradiated and irradiated carrots.

Carrots, variety 'Touchon', grown at Lammefjord or at St. Jyndevad were used for the quantitative determination of the calcium content, whereas good carrots of an "unknown" variety from the greengrocer were used for the experiments concerning the movement of calcium into the tissue.

The cork/periderm layers and the adjacent four layers of the p.p. tissue of approximately 0.5 mm thickness were analyzed separately for calcium content from each of five carrots at each treatment. The cork/periderm layers were scraped off with a knife and the other layers were cut off with a razor parallel to the surface of the carrot. The tissues were transferred to closed weighing glasses immediately after cutting and analyzed for dry matter and calcium.

Whole carrots were immersed in distilled water or in a 0.2% $CaCl_2,2H_2O$ solution for two hours, rinsed with water, blotted and analyzed for calcium content. The rinsing water was analyzed for calcium in order to calculate the $CaCl_2$ film left on the surface.

The calcium content was determined in a Jarrell-Ash Atomic Absorption Unit, i.e. a spectrophotometer similar to a flame photometer except that it measures energy as it is absorbed by atoms rather than as it is emitted by atoms. A light source of the characteristic wavelength (422.6 nm for calcium) is directed through the flame with the atomized sample. The amount of energy absorbed by the flame is proportional to the concentration of the element in the sample. The lower limit for detection of calcium in plant tissues is 0.2 ppm (Anonymous 1965b).

Cylinders were cut off parallel to the surface with a 12 mm cork-borer for determination of the translocation (fluxes) in the p.p. tissue of the "tissue calcium" and externally applied calcium. Some cylinders were cut with undisturbed surfaces (cork/periderm layers) and others without. The tissue cylinders were immediately immersed for two hours in 0.2% $CaCl_2,2H_2O$ with 0.1 uCi $^{45}CaCl_2$/ml added. After rinsing with the same concentration of inactive $CaCl_2$ and blotting, the cylinders were placed in a hand microtome (Sartorius No. 24) and 0.25 mm disks were cut off right-angled to the surface with a very sharp razor that was always pushed with uniform pressure from surface side towards the centre. The disks were fixed to microscope slides by a film of albumen-glycerol and freeze-dried overnight. This process caused a uniform shrinkage of 0.7 mm of the disks. The disks were then exposed with a uniform pressure to an X-ray film.

The carrots were non-irradiated or treated with 12 or 25 krads γ-rays in the ^{60}Co plant at Risø.

4.2.2. Results and Discussion

The carrots were not fully turgescent and absorbed water when immersed (Table 15), which gave a uniform basis for determination of the calcium content. The weight of the carrots varied from 103 to 203 g with an average of 151 g, and the weight increase during immersion varied from 0.22 to 3.65 %, obviously without any connection with size, and on an average approximately 0.5%. This is not a large value when based on whole carrots, but for the outer layers of the carrots it is considerable (Table 15); as such it may have a significant effect on the Ca^{++} uptake or release (compare the discussion in paragraph 2.2.1.2 of the water loss and water uptake in carrots).

There was about double as much calcium in the cork periderm layers scraped off as there was in the next 0.5 mm of the p.p., and the decrease proceeded at a considerably lower rate in the depths of the tissue (Figure 4). The difference between comparable curves regarding the depth was in all cases highly significant. Regarding the treatments, there was a significantly ($P < 0.01$) lower calcium content in the irradiated than in the non-irradiated water-absorbed tissue (curves I and II) and a significant ($P < 0.05$) uptake of calcium ($CaCl_2$) in the irradiated tissue compared to water-absorbed irradiated tissue (curves II and IV), but not compared to the $CaCl_2$-immersed non-irradiated tissue (curve III), nor between the water-absorbed and the $CaCl_2$-immersed non-irradiated tissues (curves I and III). These results mean that there were significant amounts of calcium released from the carrot cork/periderm layers and p.p. when immersed in water after irradiation with 12 krads (curve II vs. curve I), and that there was a significant net uptake of calcium when the irradiated carrots were immersed in 0.2% $CaCl_2,2H_2O$ (curve IV vs. curve II). The uptake was highest in the cork/periderm layers and with a steeper decrease inwards in the tissue than for the other treatments (curve IV). The net uptake of calcium in the non-irradiated tissue was insignificant.

The results of this investigation - made in 1965/66 - are in close agreement with those of Echandi & Massey (1970), who found a considerable loss of electrolytes, including Ca^{++}, already after 10 krads treatment of carrot tissue (cf. also Skou 1963 I).

Table 15. Dry matter content in per cent in different depths of carrots.

Treatment[1]		Cork/ periderm layers	0.5 mm thick layers of the phloem parenchyma towards the centre				Average ±SE	Significant difference between layers
			1st	2nd	3rd	4th		
	Lammefjord:							
I	In water for two hours	12.2	11.8	11.8	11.6	11.6	11.8±0.48	ns[2]
II	ditto and 12 krads	12.5	12.5	12.3	12.2	12.3	12.4±0.31	ns
III	0.2% $CaCl_2,2H_2O$ for two hours	12.5	11.4	11.5	11.7	11.7	11.8± .40	ns
IV	ditto and 12 krads	12.2	12.1	12.3	12.3	12.3	12.2±0.56	ns
	washed	15.8	14.5	14.2	14.0	13.8	14.4±0.46	*
	St. Jyndevad:							
	washed	13.9	11.6	11.2	11.3	11.3	11.9±0.74	(*)

[1] The treatments marked with Roman numerals refer to Figure 4.

[2] ns = no significance; * = significance on the 5% level.

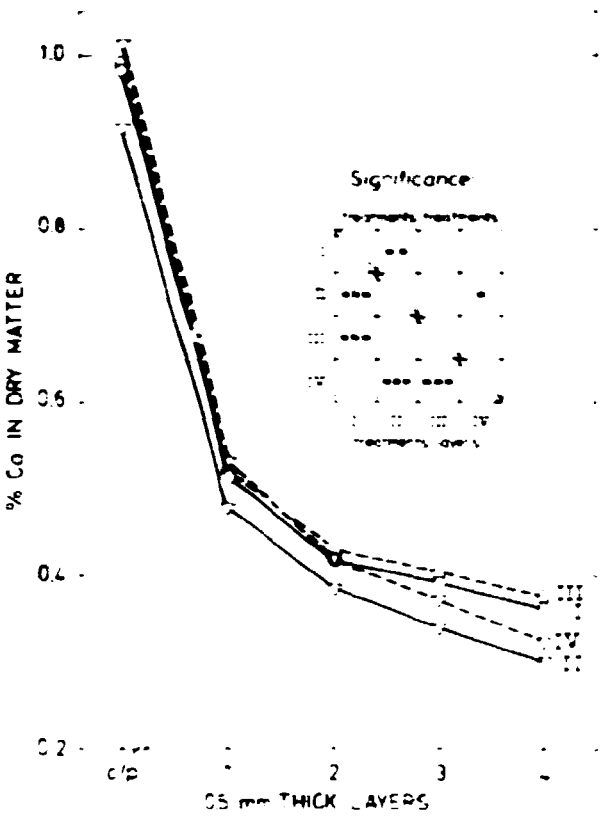

Figure 4. The calcium content of the outermost part of the carrots as measured in 0.5 mm thick layers taken from the outside inwards after different treatments. c/p: the cork/periderm layers; ○ : non-irradiated; ▽ : irradiated with 12 krads; ——: carrots immersed in water for two hours (I and II); – – –: carrots immersed in 0.2% $CaCl_2$, $2H_2O$ for two hours (III and IV); I-IV the four sets of treatments given in Table 15; i: incomparable treatments. The SE is ±0.032%. Variety, 'Touchon' from Lammefjord.

A release of calcium was found from both non-irradiated and irradiated whole carrots when immersed in water, but no significant difference was detected in the amount of calcium in the water under the conditions used.

The overall content of calcium in the carrots used is close

to that noted in the literature (see above). The relatively high amount in the surface layers and the decrease in the depths of the p.p. were not caused by the high level of exchangeable calcium in the Lammefjord fen soil of marine origin as carrots from the sandy St. Jyndevad soil had the same gradient - possibly at a lower level (Figure 5 and Table 16, cf. Skou 1971a VIII).

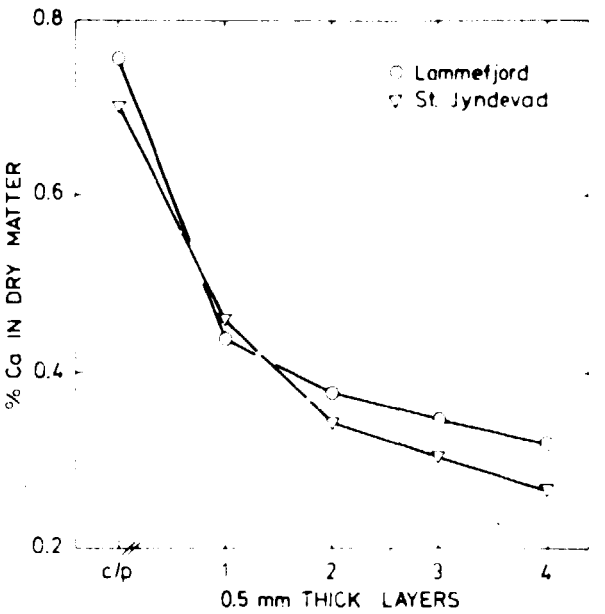

Figure 5. The calcium content of the outermost part of rinsed but otherwise untreated carrots from two very different soils. c/p: the cork/periderm layers. 1-4 constitute 0.5 mm thick layers taken from the outside inwards. Variety, 'Touchon'.

Table 16. Exchangeable calcium and pH in soil from the carrot fields.

Locality	Type of soil	pH	Exchangeable Ca^{++} meq./100 g soil
Lammefjord	Fen soil of marine origin	7.7	35.3
St. Jyndevad	Sandy soil	6.5	5.5

The ^{45}Ca autoradiogram shows that practically no calcium is accumulated in or passed through the undisturbed non-irradiated cork/periderm surface (Figure 6), whereas accumulation of Ca^{++} in the cork/periderm is evident after irradiation, but without any visible difference between 12 and 25 krads (Figure 6). Only very little calcium has penetrated further into the tissue past these surfaces. On the other hand, Ca^{++} accumulated in and penetrated further into the depth of the tissue when exposed by cutting, and no difference was observed between non-irradiated and irradiated disks (Figure 6, all the cut surfaces). The picture resembles comparable results on Ca^{++} movement in apple tissues (Collins & Wiley 1967).

The stripes seen on most of the disks indicate an easier passage for Ca^{++} in the medullary rays radiating from the stele into the phloem parenchyma. These stripes cannot be caused by contamination at cutting as the clean razor was in all cases drawn with a uniform pressure from the surface side of the tissue cylinder towards the stele.

The cause of the apparent difference between the quantitative measurement of calcium uptake and movement in the tissue through the cork/periderm surface and the autoradiogram may be that, for the latter, undisturbed surfaces without root traces were carefully selected whereas the larger quantities used for the former could not be selected in this way.

The significant dry matter gradient found in untreated carrots was lost as they reached full turgescence in two hours when immersed in water (Table 15). This confirms the known fact that carrots are not well protected against water loss and water uptake through the cork/periderm layers (cf. e.g. van den Berg & Lentz 1968, van den Berg & Yang 1969, Apeland & Baugerød 1971, Djacenko 1971). In the presence of 0.2% $CaCl_2,2H_2O$ there was at the same time a significant calcium uptake in the outermost parts of the carrot tissue exposed by disturbed or wounded (cut) cork/periderm layers. This is in agreement with the note of Poole (1976) about absorption of Ca^{++} in carrot tissue, and it has nothing to do with the known phloem immobility of Ca^{++} (cf. e.g. Ziegler 1975). There was hardly any transport of calcium through undisturbed cork/periderm layers.

In irradiated tissue, however, calcium was more easily

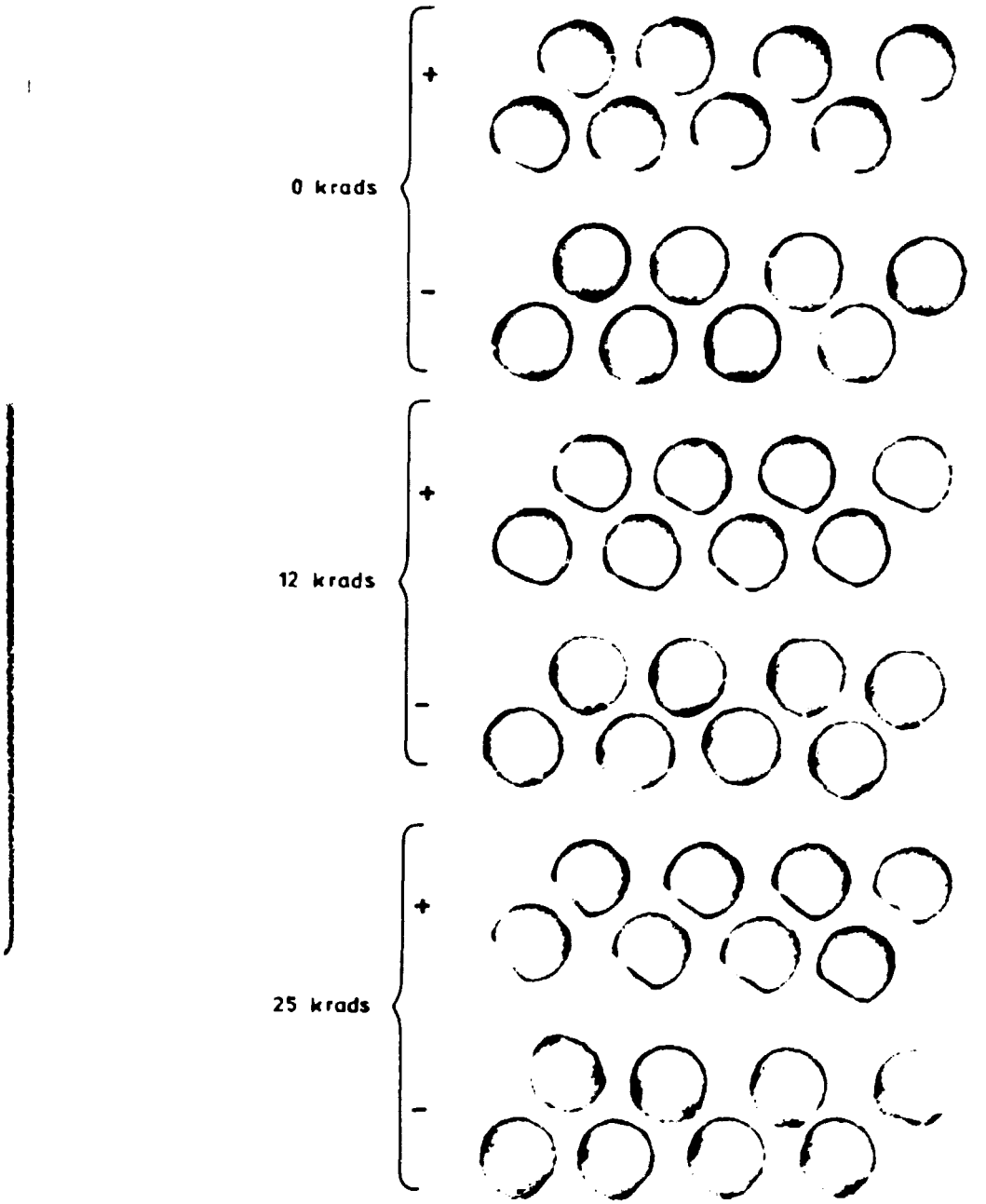

Figure 6. Text on page 67.

translocated so a significant release of calcium from the tissue was detected when immersed in water and a significant uptake of calcium, too, in the tissue in the presence of 0.2 $CaCl_2,2H_2O$ - first and foremost in the cork/periderm layers - and with a steeper gradient in the p.p. tissue than for the other treatments. These findings indicate a simultaneous outward translocation of calcium, such as occurred when the tissue was immersed in water.

The excess of calcium demonstrated in the surface tissue in these experiments, especially in the case of irradiated carrots, may act as a protection against microbial attack because of the inhibition of the activity of the pectolytic enzymes and precipitation of the oxalic acid produced by the pathogens. This should be compared to Bateman (1964) and Hancock & Stanhellini (1968) who demonstrated a protective effect of accumulated calcium in the wounds.

Rinsing after treatment, and analysing the water for calcium content, showed that 1.57 · Ca^{++}/cm^2 remained on the surface of the carrots. Calculated as $CaCl_2,2H_2O$, its specific gravity considered, it constitutes a $2.3 \cdot 10^{-2}$ cm thick layer when dried up on the carrots. $CaCl_2$ does not, however, dry up to this extent, and the layer cannot be regarded as uniform for which reason there will be much less $CaCl_2$ on several areas. Therefore, though high concentrations of $CaCl_2$ inhibit the growth of *B. cinerea* and *S. sclerotiorum* (see above and Skou 1971a VIII), it is very unlikely that $CaCl_2$ itself left on the surface has any inhibitory effect on the growth of the fungi.

Figure 6. Autoradiogram that shows the accumulation on and penetration of calcium into the phloem-parenchymatous tissue of carrots during two hours immersion in 0.2% $CaCl_2,2H_2O$ with 0.1 µCi $^{45}CaCl_2$/ml added. Six days of exposure to X-ray film. The rows of disks marked + have an undisturbed plane surface (cork/periderm) at the lower left. The disks marked - are cut deeper and have no surface layers. The picture is slightly reduced relative to the size of the freeze-dried disks.

4.3. Calcium and Microorganisms

In microorganisms, calcium may be regarded as a micronutrient, but the requirement is difficult to demonstrate because it amounts to only a few ppm. Apart from being a cofactor for some enzymes, the exact function of calcium in the cells of microorganisms is unknown. It is, however, suggested that it is required for the formation of proteases (see Nicholas 1963), or it can replace other divalent cations stimulating the production of endo-PG (Mussell 1973).

In *Aspergillus niger* v. Tiegh. and *Fusarium oxysporum* Schlecht. ex Fr. the omission of calcium had no effect, whereas lack of calcium in the growth medium strongly reduced the growth of *Rhizoctonia solani* Kühn and *Sclerotium rolfsii* Sacc. The reactions of other fungi lay in between these two extremes (Steinberg 1948). Calcium may also stimulate growth of some other microorganisms and higher fungi, and it is shown to be required for nitrogen fixation. In experiments on agar medium with the cultivated mushroom (*Agaricus bisporus*), Treschow (1944) found that there is an antagonistic effect of calcium against potassium and magnesium, and that the physiological effect of calcium depends on the concentration of other salts. The mushroom has an absolute requirement for calcium and no inhibitory effect was observed until concentrations above 0.005M $CaCl_2$. This is analogous to the present author's findings with *B. cinerea* and *S. sclerotiorum* (cf. Skou 1971a VIII). Later, the interaction of calcium with magnesium and its antagonism to the potassium ion was verified by Nicholas (1963).

The addition of small amounts of $CaCl_2$ to malt extract (1.6%) agar medium for *B. cinerea* and *S. sclerotiorum* had a weakly stimulating effect. The inhibitory effect of higher concentrations of $CaCl_2$ in the medium could just as well be a salt effect, or that of the osmotic pressure (cf. Bollard & Butler 1966), as a toxic effect of the Ca^{++}-ion (Skou 1971a VIII).

4.4. Summary

The radiation-induced degradation of macromolecular cell wall constituents increases with the dose. The degradation of pectic substances is measurable at the sprout inhibitory level from about 4 krads and higher doses, and at the same level of

irradiation an increased permeability of cells and plant tissues becomes detectable. The release of electrolytes from the tissues obviously increases exponentially with the irradiation dose, and Ca^{++} is the cation that most readily leaches from the irradiated tissue. On this basis it is supposed that there is a connection between the degradation of pectic substances and the increased mobility of calcium in the irradiated tissue. This is supported by the radiation-induced decarboxylation of pectic substances indicating that electrovalent links with calcium are broken.

The effects of calcium on plant tissues are multitudinous. Calcium is of importance for the cell permeability, which it contributes to stabilize, but at the same time the direct action of calcium results in a decreased cell permeability; an effect that is probably related to the binding with phospholipids in the cell membranes.

The linkage between calcium and the carboxylic groups of the pectic substances increases the rigidity of the cell walls, but calcium also contributes to the build-up of the elasticity of the cell walls. These facts constitute the basis for the practical use of calcium to increase the firmness of plant tissues - a treatment that has also given promising results in refirming irradiated plant products.

Calcium-deficient plants are more susceptible to diseases than plants receiving normal amounts of this nutrient. The reason for this may be that the pectic substances of such plants are more easily hydrolyzed by the pectolytic enzymes produced by the pathogens.

The oxalic acid produced by some pathogens may remove calcium from the pectic substances in the middle lamellae and thereby make them more easily hydrolyzable by the enzymes.

In this connection it should be mentioned that disease resistance in some plants seems connected with a higher calcium content or with the accumulation of calcium in the wounds. Further, various experiments have show that external application of calcium may or may not exhibit a protective effect against diseases.

These facts constitute the basis for the present author's investigations. Double as much calcium was found in the cork/periderm layers as was found 0.5 mm deeper into the phloem paren-

chyma, from where there was a smaller gradient further into the tissue. The overall content of calcium found in the carrot tissue is in agreement with the majority of reports in the literature.

Further, the results revealed a significant calcium release from the cork/periderm layers and the outer parts of the phloem parenchyma tissue when immersed in water after irradiation with 12 krads, and a significant uptake of calcium when the irradiated carrots were immersed in 0.2% $CaCl_2,2H_2O$. For the non-irradiated carrot tissue, the release was insignificant.

^{45}Ca autoradiograms showed practically no accumulation in or passing through the undisturbed non-irradiated cork/periderm surface, whereas $^{45}Ca^{++}$-ions accumulated after irradiation with 12 or 25 krads. Only very little calcium penetrated further into the tissue past these surfaces. Only when the tissue was cut did the calcium penetrate further into the depths of the tissues, without visible differences between irradiated and non-irradiated specimens. This result corresponds with the higher calcium content found in wounds, which may act as a protective measure against pectolytic enzymes and oxalic acid produced by microorganisms. This feature may contribute to the protective effect of $CaCl_2$ and explain why the effect is absent after very gentle handling of the carrots as shown in Chapter 2.

It is unlikely that calcium has any inhibitory significance for the growth of pathogens such as *B. cinerea* and *S. sclerotiorum*, because the amount left on the carrots after application is too small, and even though the concentration increases when it dries out as a film on the surface, the layer is too thin and unevenly distributed.

These facts constituted the background for a series of experiments intended to determine if treatment of carrots with calcium could compensate for the reduced resistance of the tissues after irradiation, thereby giving a protective effect.

5. PECTOLYTIC ENZYMES AND THEIR ACTIVITY

5.1. Definition of Pectic Substances

It is a difficult task to give an exhaustive description of the complex pectin molecule or pectic substance and its different degradation units (Rombouts 1972, Albersheim 1973, Aspinall 1973, Lamport 1973, Bateman & Basham 1976). Throughout this paper the pectic substances are defined according to their main characteristics when used as substrates for the pectolytic enzymes, which definitions are also those usually occurring in the literature.

Protopectin is defined as the pectic substances that remain insoluble in the plant tissue after extraction with water. Its units are probably cross-linked with cations such as calcium or magnesium.

Pectin refers to water-soluble, highly methoxylated pectic substances, whereas pectinic acid is the molecules with a lesser degree of methoxylation between the pectin and the pectic acid. Pectic acid is the non-esterified polygalacturonic acid (cf. e.g. Wood 1955a).

The exo-type of pectolytic enzymes principally liberates galacturonic acid from the end of the molecular chain, whereas the endo-type mainly splits the molecule at random into a number of oligomers that may be further degraded by the same or other enzymes. The use of the above prefixes in this way in enzymology and biochemistry makes for confusion with biology, where these terms are used for enzymes that exudate from living cells or for those that remain in the cells and only leak out from ruptured cells or autolyzing dead cells, respectively. To cover the latter situation, the terms exo- and endo-cellular enzymes will be used throughout the paper.

5.2. The Pectolytic Enzymes

There are three principal types of pectolytic enzymes, namely (1) pectinesterase (PE), (2) polygalacturonase (PG), and (3) pectin lyase (PL).

PE (3.1.1.11.*, pectin pectyl-hydrolase (Anonymous 1965a),

*International recommended numbering (Anonymous 1965a, Koller 1966).

also called pectin methyl esterase) hydrolyzes the methyl ester groups of pectin (pectin + n H_2O = n methanol + pectin, cf. Figure 7).

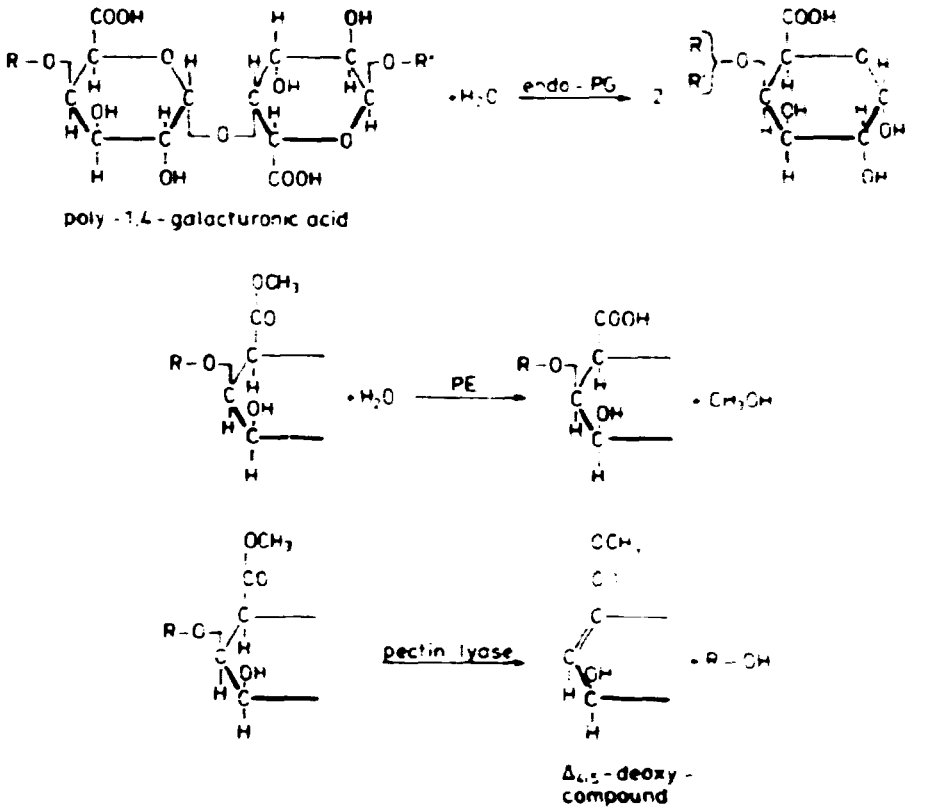

Figure 7. Schemes of reactions promoted by the pectolytic enzymes. Endo-polygalacturonase (endo-PG) principally reacts on poly-1,4-galacturonic acid more or less at random along the molecular chain. Pectinesterase (PE) hydrolyzes the methoxyl groups on the galacturonic acid units. Pectin lyase (PL) splits the molecular chain by transelimination, which results in the formation of a $\Delta_{4:5}$-deoxy compound. R and R' are hydrogen, or a smaller or larger pectic chain of galacturonic units.

PG (3.2.1.15., poly-α-1,4-galacturonide glycanohydrolase (Anonymous 1965a), also called pectin depolymerase or pectinase)

hydrolyzes α-1,4-D-galacturonide links in pectate more easily than in pectin. Polymethylgalacturonase (PMG) behaves oppositely. Both PG and PMG occur in exo forms (exo-PG (3.2.1.40.) and exo-PMG(-); Koller 1966) that hydrolyze the molecular chain from the end, and in endo forms (endo-PG (3.2.1.15) and endo-PMG (3.2.1.41.); Koller 1966, cf. Figure 7) that hydrolyze the molecular chain more or less at random. PG was previously the name for the enzymes that disintegrated the polygalacturonic acid chain of the molecule. To-day, the name refers to the hydrolytic enzymes that prefer pectate to pectin as substrate (cf. e.g. Wood 1955a), or it is used in cases where the preferred substrate of the enzyme in question is unknown (cf. Figure 7).

Pectin lyase (PL,-) and pectate lyase (PAL, 4.2.2.2., Anonymous 1965a) denote whether the enzyme in question prefers pectin or pectate as substrate. PL and PAL are poly-α-1,4-D-galacturonide lyases, also called transeliminases, which eliminate $\Delta_{4:5}$-D-galacturonate residues from pectin and pectate, respectively, thus bringing about depolymerization. As in the case of polygalacturonases, PL and PAL exist in exo forms (exo-PL(-) and exo-PAL (4.2.2.2.); Koller 1966) and in endo forms (endo-PL (4.2.2.3.) and endo-PAL (4.2.2.1.); Koller 1966, cf. Figure 7).

5.3. Review of Methods for Measuring the Activity of Pectolytic Enzymes

5.3.1. Extraction of Enzymes from Healthy and Diseased Tissues

In cases of soft-rot, the enzymes were often easily extracted by squeezing the juice of the rotted tissue through layers of cheese cloth and by a simple filtration (cf. e.g. Fernando & Stevenson 1952, Cole 1956, Bateman 1963, Spalding 1969, Stephens & Wood 1975), or the juice was expressed by a screw press (Cole & Wood 1961). In other cases, the tissue was disintegrated in a Waring Blender or ground in a mortar under various conditions (see below).

In a few cases the enzymes were extracted with water (cf. e.g. Elarosi 1958, Bateman 1963, 1964, Bateman & Beer 1965).

NaCl was most extensively used as extraction medium in different concentrations from 0.2M to ca. 2.5M (10%) (cf. e.g. Hertesz 1955, Cole & Wood 1961, Ayers & Papavizas 1965, Ayers, Papavizas & Diem 1966, Hancock & Millar 1965, Hancock 1966, 1976, Byrde & Fielding 1968, Swinburne & Corden 1969, Bugbee 1972, Stephens & Wood 1974). The extractions were often made with solutions buffered with phosphate (Mason 1955, Cole & Wood 1961, Bugbee 1972, El-Goorani, Abo-El-Dahab & Khoshnaw 1976, Mollenhauer, Byrde & Fielding 1968) or tris-HCl (Hancock 1968, Papilli, Mazzucchi & Pierini 1976) at different pH levels. Further, it was sometimes necessary to add such substances as cysteine (Mason 1955, Hancock 1968), dithionate (Byrde & Fielding 1968) or polyvinylpyrrolidone (Swinburne & Corden 1969, Spalding & Abdul-Baki 1973) in order to protect the enzymes against inactivation by phenolics and their degradation products. The present author used the method of squeezing the juice of rotted carrots through layers of cheese cloth.

5.3.2. Purification of Enzymes or Enzyme Preparations

The extracts or culture fluids were filtered and/or centrifuged in order to get rid of cells or cell remains. The resulting crude enzyme preparations were used directly for determination of activity (cf. e.g. Mount, Bateman & Basham 1970, English, Jurale & Albersheim 1971) as did the present author, or for purification.

Dialysis against distilled water or weak salts and/or buffer solutions at an appropriate pH was the most simple purification measure (cf. e.g. Deshpande 1959, Bateman 1963, Hancock, Millar & Lorbeer 1964, Hancock & Millar 1965, Bateman & Beer 1965, Hancock 1968, Hsu & Vaughn 1969, Hall & Wood 1973), or the first step in further purification. Often the first step was salting out the proteins with fractioned precipitation by different concentrations of $(NH_4)_2SO_4$ (cf. e.g. Hall & Wood 1970, 1973, Garibaldi & Bateman 1971, Spalding & Abdul-Baki 1973, Stephens & Wood 1974, Hancock 1976), or by precipitation by acetone (Spalding 1969). Further steps were dialysis or gel filtration on columns of variously specified Sephadex resins, and on DEAE-cellulose or carboxymethylcellulose (CMC) (cf. Albersheim & Killias 1962, Byrde & Fielding 1962, 1968, Edstrom & Phaff 1964, Albersheim 1966, Dean & Wood 1967, English et al. 1972, Mussell

& Strouse 1972, Hall & Wood 1973, Spalding & Abdul-Baki 1973, Stephens & Wood 1974, 1975, Cooper & Wood 1975, Hancock 1976, and others). The use of other adsorbing substances may be valuable in the purification; for example, diatomaceous earth or alginic acid (Kertesz 1955, Waggoner & Dimond 1955), 'Bio-Rex 70' column (English et al. 1972), and ion-exchange chromatography by adsorption on 'Duolite CS-101' (Swinburne & Corden 1967, 1969). For separation of the single enzyme compounds, isoelectric focusing has been of great value (cf. e.g. Mussell & Strouse 1972, Mussell 1974, Stephens & Wood 1975, Hancock 1976). Other methods used for purification were paper partition chromatography (Elarosi 1958) and disc-gel electrophoresis (Barash & Eyal 1970, Basham & Bateman 1975a).

5.3.3. Effect on Plant Tissues

5.3.3.1. Permeability. Changes in this parameter during pathogenesis must be regarded as just as important as any other parameter changes. Not until the last ten or twenty years has much attention been paid to it (Wheeler & Hancey 1968). In his classical works, Thatcher (1939, 1942) showed how several pathogens were able to increase the permeability of the host tissue for some distance ahead of the hyphae. He studied the osmotic values of host and pathogen cells and used the plasmolysis/deplasmolysis test for determination of the permeability to water, urea, dextrose and other solutes. His results are discussed under *Botrytis cinerea* and *Sclerotinia sclerotiorum* below, and the method has been used by others (cf. e.g. Hancock 1968, Stephens & Wood 1974).

The increased permeability of tissues or cells, due to physical treatment or microbial attack, leads to the loss of electrolytes, which may be measured indirectly by the increase in the conductivity when immersed in water (cf. e.g. Skou 1963 I, Hall & Wood 1970, 1973, Byrde et al. 1973), or by direct determination of the amounts of ions, e.g., amino acids and cations, leaching out (Echandi & Massey 1970). These methods were improved by the use of ^{86}rubidium as a tracer element (Mount, Bateman & Basham 1970, Basham 1974, Basham & Bateman 1975a, b).

The electrolyte method may be used in connection with the

blotting and weighing method (Sk a [illegible]), Salisbury & [illegible] 1969, Hall & Wood 1970, Mussell & Strouse 1971, Basham & Bateman 1975a), or with maceration and respiration (Spalding [illegible]).

5.3.3.2. Maceration. Maceration, i.e. disintegration by loss of coherence between cells in the plant tissue, has been used as a measure of pectolytic activity since the time of de Bary (1886). Brown (1915) developed a simple and rapid method for measuring the rate of maceration. Pieces of tissue were immersed in the test solution, and the time that elapses for cell coherence to be lost to such an extent that cells can be pulled apart without perceptible resistance is tested by hand, pincet or spatula. The degree of softness is given by scores using a 0-5 index (cf. e.g. Brown 1915, Hannan 1955, Bateman 1968, Mount, Bateman & Basham 1970). Though subjective, the method has been regarded as useful and has been used in different modifications ever since it was developed (see, e.g., Bateman 1968). One of the modifications that removes the subjective element is the turbidity procedure (Bateman & Beer 1965, Bateman 1968) in which the tissue is placed in solution on a wrist-action shaker at standardized conditions, and the turbidity of the resulting cell suspension is measured on a spectrophotometer at 475 nm relative to known cell suspension. The chlorophyll in the freed cells may also be used as a measure for the macerating activity (Zathlin & Coltrin 1964). Mechanical instruments have not been used to any greater extent for measuring the tension or hardness of tissues exposed to pectolytic enzymes (McClendon & Somers 1960, Sherwood 1966), such as is the case for the quality of vegetable products and in connection with irradiation, which is discussed in Chapter 7.

5.3.3.3. Cell Death. Investigations have centered on cell death in connection with pectolytic activity ever since it was first observed in the microscope by de Bary (1886), who established that not all the cells in the exposed tissue had the normal ability to plasmolyze and that there was a weak swelling of the cell walls. Brown (1915) confirmed these findings and the relationship between maceration and cell death. Tribe (1955) developed the outstanding neutral red method (NR-index method), which is based on the ability of living cells to plasmolyze and

retain the neutral red stained, whereby they stain but red in colour. Dead cells have lost this ability and stand out colourless or weakly pink. To the author's knowledge, this classic method, with some slight modifications, is still almost the only method used for determination of cell death in plant tissues (Cole 1956, Hall & Wood 1970, 1973, Wood 1972, Byrde, Fielding & Archer 1973, Bashan & Bateman 1975a, b, Stephens & Wood 1975, Bateman & Bashan 1976).

5.3.4. Methods for Measuring the Enzyme Activity on Pectic Substances

The enzymes used for the experiments were produced either on susceptible hosts or in media suitable for the purpose and the particular organisms in question. Often the culture media were supplied with enzyme-inducing substances, as in several cases the pectolytic enzymes were produced almost only. Crude enzymes prepared from extracts of diseased plant tissue or from culture filtrates were used for the assays without further treatment, or they were purified and separated from accompanying pectolytic enzymes to a smaller or greater extent. In older investigations the use of crude enzymes was common practice, and for some purposes these methods were used until recently (cf. e.g. El-Goorani, Abo-El-Dahab & Khishnow 1976), although purification methods have improved. The present author used crude enzymes, because the aim of the experiments was to elucidate the effects of the enzymes, as they are produced by the pathogens, and as they act and interact during the pathogenesis.

5.3.4.1. Pectate Gels and Cup-Plate Assays. Organisms are easily screened for pectolytic activity by pectate gels (Dowson 1957, Paton 1959, Gehring 1961/62) and by the cup-plate assay, which may give a rough estimate of the amount and type of enzymes in question, but it is not specific for any single enzyme of the group (Dingle, Reid & Solomons 1953, Nagel & Vaughn 1961, Swinburne & Corden 1967, Howell 1975, Puhalla & Howell 1975). The effect of salts and cations may easily be detected by the cup-plate assay (Dingle, Reid & Solomons 1953), as may the acid produced by hydrolysis of the methoxy groups using methyl red as indicator.

5.3.4.2. Viscosity. The depolymerization of pectic substances is frequently determined by viscosimetry using different-sized viscosimeters (Cannon-Fenske, Fenske-Oswald, Höppler). Though not specific for any pectolytic enzyme, the method is very valuable as a measure of enzyme activity, and in connection with analysis of the degree of hydrolysis, or the trans-eliminative split, it gives extensive information about the more or less random depolymerization from the end (exo-) of the molecule or from inside (endo-), even though there is no simple relation between viscosity and molecular size.

Generally the results are given as the decrease in relative viscosity, or as the per cent reduction in viscosity per unit of time (cf. e.g. Gäumann & Böhni 1947a, Bell 1951/52, Wood 1955, Nagel & Vaughn 1962, Bateman 1963b, Bateman & Beer 1965, Ayers & Papavizas 1965, Ayers, Papavizas & Diem 1966, Tani & Nanba 1969, Mussell & Strouse 1972, Cooper & Wood 1975, Lisker, Katan & Henis 1975).

5.3.4.3. Pectinesterase Assays. The PE hydrolyzes the methoxyl groups of pectin or pectinic acids and liberates methanol and carboxyl groups, both of which may be used as a measure of PE activity. Titration of the free carboxyl groups with weak alkali is the most widely used method, either by determining the acid produced in a fixed time (Gäumann & Böhni 1947a, Smith 1958a, Bonnet & Venard 1975), or by continuous titration (cf. e.g. Jansen & McDonnell 1945, Kertesz 1951, 1955, Winstead & Walker 1954, Echandi & Walker 1957, Cole & Wood 1961, Ayers & Papavizas 1965, El-Goorani, Abo-El-Dahab & Khoshnow 1976). Sometimes the method is combined with Ca-pectate precipitation (see, e.g., Gäumann & Böhni 1947a), or the acid production may be determined manometrically by measuring the amount of CO_2 evolved from $NaHCO_3$ added to the reaction mixture (Nagel & Vaughn 1961). The methanol liberated may be determined by colorimetry (Boos 1948, Wood 1955, Jansen, Jang & Bonner 1960) or by gas-chromatography (van den Berg & Yang 1969). The present author mainly used the method of continuous titration, but in connection with determination of the activity of polygalacturonase and pectin lyase, the simultaneous activity of pectinesterase was measured by determining the acid produced in a fixed time.

5.3.4.4. Polygalacturonase Assays. The polygalacturonase (PG) activity may be measured as the equivalents of reducing groups (aldehyde groups) formed by hydrolytic degradation of the molecular chain of pectic substances. In combination with viscosimetry the method may reveal whether the enzyme in question is of exo- or endo-type.

Fehling's reaction may be used as a rapid qualitative test for the formation of reducing groups in reaction mixtures or in growth media with non-reducing carbon sources, e.g. pectic substances, added (Matus 1948, Veibel 1954), and for these purposes the method was used by the present author. Paper chromatography is another qualitative method used for the detection of reducing substances (Hancock & Millar 1965).

The modified Willstätter-Schudel hypoiodite method or the Schoorl's iodine method has been frequently used for the quantitative determination of the reducing groups (cf. e.g. Jansen & McDonnell 1945, Phaff 1947, Matus 1948, Kertesz 1951, 1955, Echandi & Walker 1957, Patel & Phaff 1959). The present author also used this method. In recent years, however, it has been replaced to some extent by the dinitrosalicylic acid method (cf. e.g. Millar 1959, Hancock & Millar 1965, Bateman & Beer 1965, Bateman 1966, Barash & Eyal 1970). The basis for both methods is the oxidizing of the reducing groups. In the former methods the excess iodine is titrated with $Na_2S_2O_3$ in order to find the amount reduced during the reaction, normally using a starch indicator. In the latter method the reduced dinitrosalicylic acid (3-amino-5-nitrosalicylic acid) is determined directly by spectrophotometry at 575 nm. An arsenomolybdate procedure has also been used for determination of the accumulation of reducing compounds (Basham & Bateman 1975a).

The decision whether the enzyme in question is a pectin polygalacturonase (polymethylgalacturonase, PMG), or a pectate polygalacturonase (PG), is based on the relative reaction rate on pectin and pectate, respectively.

Although the reducing group methods are still in use to-day, they are often used together with or replaced by the thiobarbituric acid method (TBA, 2-thiobarbituric acid) or the TBA-periodate method in various modifications (cf. e.g. Neukom 1960, Albersheim, Neukom & Deuel 1960, Preiss & Ashwell 1963, Ayers, Papavizas & Diem 1966, Sherwood 1966, Bugbee 1972, 1975). Thio-

barbituric acid forms a coloured substance with an absorption maximum at 515 nm with the hydrolysis products of the PG activity. A modification of the former TBA method was used by the present author.

By combining these methods with the cup-plate method, the maceration method, viscosimetry or paper chromatography of the mono- and oligomer reaction products (cf. e.g. Patel & Phaff 1959, 1960, Nagel & Vaughn 1961, Endo 1961a, 1964, Ellstrom & Phaff 1964b, Hancock, Millar & Lorbeer 1964, Ayers & Papavizas 1965), it is possible to distinguish between the exo- and endo-type of the enzymes (Singh & Wood 1956, Endo 1961b, 1964, Byrde & Fielding 1962, 1968, Bateman 1966a, Papavizas & Ayers 1965, Bugbee 1972, 1975, Mussell 1973, Cooper & Wood 1975).

Preiss & Ashwell (1963) determined the activity of PG by an orcinol assay for the remaining polygalacturonic acid based on the fact that galacturonic acid is destructed by alkali, while the deoxy-compound is stable. The non-existence of the latter reveals that PG has hydrolyzed the pectic substance.

5.3.4.5. Lyase Assays. The activity of pectin lyase (PL) and pectate lyase (PAL) may be measured by employing TBA and TBA-periodate, which form a coloured substance with absorption maximum at 547 nm with the unsaturated reaction products (cf. e.g. Albersheim, Neukom & Deuel 1960, Ayers, Papavizas & Diem 1966, Bateman 1966, Sherwood 1966, Tani & Nanba 1969, Hancock, Eldridge & Alexander 1970, Mount, Bateman & Basham 1970, Garibaldi & Bateman 1971, Bugbee 1972, 1975, Spalding & Abdul-Baki 1973, Stephens & Wood 1974, 1975, Basham 1974, Basham & Bateman 1975a, b). The maxima, 515 and 547 nm, for the products of the PG and the PMG activities and of the lyase activities, respectively, are sharp enough to be clearly distinguishable (Albersheim, Neukom & Deuel 1960).

The unsaturated degradation products from the lyase activity on pectic substances may be determined directly without further preparation as they have specific absorption maxima in UV-light at 230-235 nm. Starr & Moran (1962) stated that pectate lyase activity results in compounds (with free carboxyl group(s)) with an absorption maximum at 230 nm, whereas the pectin lyase activity results in compounds (with methylated carboxyl group(s)) with an absorption maximum at 235 nm. These two maxima cannot be

distinguished unless the enzymes act on their respective substrates in a pure state, which is very rare. This seems confirmed in the various works as the absorption maxima indicated obviously vary by mere chance between 230 and 235 nm, regardless of which pectic substance is used as substrate (cf. e.g. Albersheim & Killias 1962, Edstrom & Phaff 1964a, b, Hancock & Millar 1965, Papavizas & Ayers 1965, Albersheim 1966, MacMillan & Phaff 1966, Hsu & Vaughn 1969, Lisker, Katan & Henis 1975, as well as most of the authors mentioned under the discussion of the TBA method because they used both methods). The procedure for determining whether it is pectin or pectate lyase, or whether they are the exo- or the endo-types of the enzymes in question, is the same as that for the PG's.

5.4. Methods for Analyses of Oxalic Acid and Calcium

The oxalic acid in plants produced by the plants or by pathogens may, e.g., be extracted with 1-2N HCl (Holden 1950, Rawlins & Takahashi 1952, Baker 1952, Maxwell 1973) or with 80% ethanol (Overell 1952), and precipitated from the extract with the calcium chloride-acetate buffer method (Baker 1952, Bateman & Beer 1965, Maxwell & Lumsden 1970, Maxwell 1973), and with the addition of $CaCl_2$ (Holden 1950) or saturated $Ca(NO_3)_2$ to the neutralized solution (Gentile 1954). After standing overnight the precipitate is centrifuged, rinsed and dissolved in H_2SO_4.

The amount of oxalic acid may then either be determined by titration with $KMnO_4$ (Holden 1950, Baker 1952, Bateman & Beer 1965, Maxwell & Lumsden 1970), or by a manometric measure of the CO_2 evolved when the oxalic acid is oxidized with $KMnO_4$ (Gentile 1954). The ethanol extracted oxalic acid may be adsorbed on an anion exchange resin, eluted with $(NH_4)_2CO_3$ and determined by paper chromatography (Overell 1952). Further, a ^{14}C tracer technique may be used (Maxwell 1973).

The free Ca^{++}-ions in the plant tissue may be extracted with water and the total amount from the ashes (Holden 1950). Extraction with hot water, weak alkali or dilute ethanol removes both the free Ca^{++}-ions and the pectin, leaving the Ca-pectate insoluble (Lokonti & Kertesz 1941, Rawlins & Takahashi 1952). Stronger treatments also bring the Ca-pectate in solution (Raw-

lins & Takahashi 1952, Henglein 1955). The Ca-pectate may, however, be extracted by oxalic acid or ammonium oxalate, which binds the cross-linked Ca^{++}-ions and leaves the pectinic or pectic acid in solution (cf. e.g. Buston & Kirkpatrick 1931, Elwell & Dehn 1939, Kertesz 1954, Henglein 1955).

The Ca^{++}-ions in solution may be precipitated by oxalate ions and the amount determined by titration with $KMnO_4$ (Henglein 1950), or it may be determined in the solution by colorimetric titration (Bateman & Lumsden 1965), or by a liquid-membrane calcium-specific electrode coupled with a millivoltmeter (Somers 1973). Finally, it should be mentioned that atomic absorption spectrophotometry was used for the determination of calcium in connection with the present author's experiments (cf. page 60; Anonymous 1965b).

The distribution of calcium in plant tissues may be detected by ^{45}Ca tracer technique (cf. e.g. Bateman 1964, Collins & Wiley 1967).

5.5. Review on the Effect of Cations on Pectolytic Enzymes

5.5.1. Pectinesterase

Apparently very little has been written about the effect of calcium on the activity of mould pectinesterase (PE). Only Lumsden (1976) noted that 0.1M NaCl or $CaCl_2$ activated the PE in dialyzed extracts of healthy bean (*Phaseolus vulgaris* L.) tissue but not in that from diseased bean hypocotyls. On PE from lucerne and orange, calcium (and magnesium) has a pronounced stimulating effect that lies beyond what can be ascribed to a simple salt effect (Lineweaver & Ballou 1945, MacDonnell, Jansen & Lineweaver 1945). These facts will be discussed in connection with the present author's results with mould PE.

Also sodium exhibits a stimulating effect on the PE activity (Lineweaver & Ballou 1945, MacDonnell, Jansen & Lineweaver 1945), but there is a clear difference between its influence on plant PE and on mould PE (McColloch & Kertesz 1947).

5.5.2. Polygalacturonases

The polygalacturonase (PG) activity is in general inhibited by divalent cations and not limited to calcium alone, though

this element has normally the greatest effect. Matus (1948), who is apparently the only research worker to have studied the effect of cations with higher valence, found that the 'pectinase' was increasingly activated with increasing valence of the cations, especially when changing from the monovalent Na^{+} to the divalent Ca^{++}, but with a further increase with Al^{+++} and a decrease with Th^{++++}. As he used commercial 'pectinase' of mould origin, and considering that these experiments took place so early, the enzyme in question need not have been a PG proper, but either a mixture or a lyase (see below). Further, Matus' (1948) experiments showed increasing activity with decreasing methoxyl content of the pectin and that the enzyme was more stable against alkali in a crude state than when purified.

Using the cup-plate method, Dingle, Reid & Solomons (1953) examined the effect of Ca^{++}-, Mg^{++}-, and Cu^{++}-ions on the PG activity of commercial 'pectinase' (Pectinol 100). Five percent $CaCl_2$ or 8% $MgCl_2$ had no inhibitory effect, while 4% $CuSO_4$ completely inhibited the enzyme activity, and 0.25% reduced it to 25% of its original activity, for which reason it was supposed that Cu^{++}-ions had a direct effect on the enzyme. It is doubtful if Pectinol 100 is a PG proper.

Mg^{++}-ions inhibited or deactivated the pectinase activity from *B. cinerea* but not from *Pythium* sp. (Chona 1932). Ashour's (1954) results concerning the effect of Mg^{++}-ions on the activity of enzymes from *B. cinerea* are in agreement with these results, but his results from *P. debaryanum* Hesse showed strongly enhanced activity using small concentrations of Mg^{++} and then a decrease using higher concentrations of Mg^{++}-ions in the reaction mixture (Figure 8). Damle (1952) found that enzyme preparations from *B. cinerea* and *P. debaryanum* were retarded by KNO_3, but more by bivalent salts such as $CaCl_2$ and $MgSO_4$; the *Pythium* enzyme being less sensitive than the *Botrytis* enzyme. In the latter, Ca^{++} and Mg^{++} seem to have a specific effect that is more pronounced for Ca^{++} than for Mg^{++}. For the *Pythium* enzyme, it may be an effect of the salt concentration only, as the inhibition enhances exponentially from about 0.0125-0.025M and up to 0.2M, whereas for the *B. cinerea* enzyme the effect of $CaCl_2$ was considerable at 6×10^{-3}M and increased steeply to about 5×10^{-2}M from where the effect levelled off. For this enzyme, the effect of $MgCl_2$ was considerable from about 2.5×10^{-2}M and

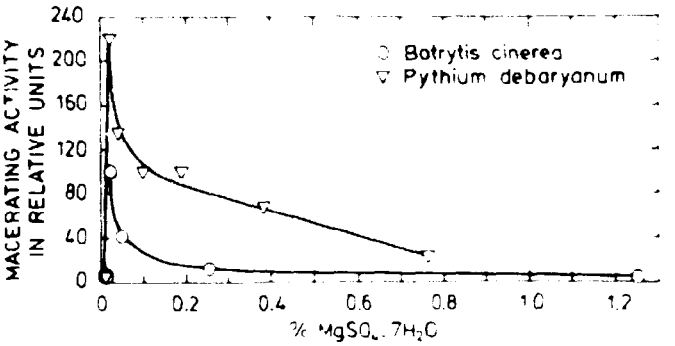

Figure 8. The macerating activity of *Botrytis cinerea* and *Pythium debaryanum* in growth media to which were added increasing amounts of $MgSO_4$. (Drawn after Ashour 1954 Tables 4 and 10).

reached the inhibitory level of $CaCl_2$ at about 0.2M. Using commercial pectinase, Zaitlin & Coltrin (1964) found Ca^{++}-ions progressively inhibitory in concentrations from 10^{-} M to above 10^{-2}M, whereas Mg^{++}-ions had no effect until about $1.5 \cdot 10^{-2}$M. At 0.1M concentrations of Mg^{++}- and Ca^{++}-ions, the activity of the macerating enzyme from *Rhizopus stolonifer* (Ehr. ex Fr.) Link was completely blocked when acting on sweet potato tissue. This effect was not reached with K^{+}-ions until 0.5M concentrations (Spalding 1969). The activity of two polygalacturonases from *Verticillium albo-atrum* Reinke & Berth. was unaffected by millimolar concentrations of Ca^{++}, Mg^{++}, EDTA, or disodium oxalate, which shows that these enzymes have no requirements for the two cations (Ayers & Papavizas 1965, Mussell & Strouse 1972).

NaCl enhances the production in vitro of pectolytic enzymes from P. *debaryanum*, whereas Ca^{++}- and Mg^{++}-ions reduce the production (Gupta 1956).

It should be mentioned here that if the production of oxalic acid from S. *sclerotiorum* on a complete medium containing 5 µM $MgCl_2$ is set at 100, a medium with 0.5mM Mg^{++} gave 86, and another with 6mM gave 98, whereas a medium without an addition of Mg^{++} gave 30 (Maxwell 1973). Thus the Mg^{++}-ion stimulates the production of oxalic acid, which acts synergistically with the PG by removing calcium from the binding to the molecular chains of the pectic substances (Bateman & Beer 1965). Further,

oxalic acid, produced by *A. niger* was shown to cause the symptoms of hypocotyl lesions of the crown rot on peanuts (*Arachis hypogaea* L.) (Gibson 1953).

The addition of $CaCl_2$ to the enzyme-pectic acid mixture inhibited the activity of commercial enzyme preparations (Manville, Reithel & Yamada 1939). Fungal PG activity was inhibited by $CaCl_2$, except in concentrations below about 10^{-3} M when added to decalcified tobacco leaf fibre. In the decalcified fibre there was an increased PG activity as compared to untreated fibre (Holden 1950). The calcium inhibition of the PG activity from *F. oxysporum* Schlecht. ex Fr. f.sp. *cucumerinum* (Sacc.) Snyd. & Hans. increased with increasing concentration and the relation between the PG activity and the calcium concentration gave a straight line in a double logarithmic plot (Corden 1965). Concentrations up to $5 \cdot 10^{-4}$ M $CaCl_2$ were without effect on the PG activity from *R. solani*, whereas higher concentrations were progressively inhibitory (Ayers, Papavizas & Diem 1966). The macerating enzyme from *S. sclerotiorum* was greatly impeded by Ca^{++}-ions (Hancock 1966).

Lumsden & Bateman (1966) noted that *Verticillium dahliae* (Berk. et Br.) Ferraris produces a Ca^{++}-stimulated hydrolase (PG) that is more reactive with pectin than with Na-pectate, and a Ca^{++}-stimulated lyase that is reactive on both pectin and Na-pectate.

Wieringa's pectate gel, based on Ca^{++} diffusion from calcium agar into a pectate layer which it renders solid, may hardly be used for determination of PG activity in any of its modifications, unless the pectins or pectinic acids used have a relatively high degree of methoxylation, in which case it may be used also for determination of pectinesterase (cf. Gäumann & Böhni 1947a, b, Kertesz 1951, Dingle, Reid & Solomons 1953, Dowson 1957, Paton 1959, Gehring 1961/62).

5.5.3. Lyases

Starr & Moran (1961, 1962) discovered a new type of pectolytic enzyme that split the molecular chain in a trans-eliminative manner producing $\Delta_{4;5}$-deoxygalacturonic acid units. The enzyme was produced by *Bacillus polymyxa* (Prazmovsky) Mig. and *Erwinia carotovora* (Jones) Bergey et al. The authors observed

that the enzyme was completely inactivated by the chelating agent, EDTA, and that the activity could be recovered by the addition of $CaCl_2$, the optimal concentration of which was found to be about 10^{-3}M.

The complete inhibitory effect of EDTA on lyases was confirmed by others. Thus the lyase from *S. [illegible]* was completely inhibited by $2 \cdot 10^{-3}$M EDTA (Ward & Walton 1961, [illegible]). The lyase activity from *Pseudomonas* sp. was inhibited to the extent of 16% in the presence of $3 \cdot 10^{-5}$M EDTA that increased to 93% at 10^{-4}M concentration (Preiss & Ashwell 1963). This indicates that small cation impurities in the reaction mixture may activate the enzyme. Such a case was, e.g., observed by Garibaldi & Bateman (1971) in that the lyase activities of crude culture filtrates, or purified enzyme fractions, from *E. chrysanthemi* Burkh. et al., were not generally stimulated by the addition of $CaCl_2$, but the activity of each fraction was completely lost in the presence of 10^{-3}M EDTA and restored by the addition of 10^{-3}M $CaCl_2$. EDTA was also inhibitory to lyases from *Corynebacterium michiganense* Bergey et al. (MacMillan & Phaff 1966), *B. [illegible]* (Ayers, Papavizas & Diem 1966), *Corynebacterium fascians* Bain & Essary (Hancock & Millar 1965), *P. [illegible]* (Mart.) Sacc. f.sp. *cucurbitae* Snyd. & Hans. (Hancock 1968, Hancock & Stanghellini 1968), and from *Arthrobacter* 547 (Rombouts 1972) in $0.5 \cdot 10^{-3}$M to $6 \cdot 10^{-3}$M concentrations. In all cases the activity was recovered by the addition of millimolar concentrations of $CaCl_2$, for which reason the absolute dependence of calcium is obvious.

Calcium was found to activate both the enzymes that split pectic substances and the macerating enzymes produced in culture by *E. carotovora* (Jones) Bergey et al. var. *carotovora* Dye. The correspondence between the macerating, chain-splitting and lyase activities was so close that it was concluded to be the same enzyme in question (Dean & Wood 1968).

The following table (Table 17) gives the effect of different concentrations of $CaCl_2$ on the activity of lyases from various microorganisms.

Results from studies of the specificity of the Ca^{++}-ion are somewhat ambiguous, though calcium always exhibits the largest effect.

Table 17. The effect of calcium concentration on the activity of lyases from various microorganisms as cited in the literature.

Organisms	Concentrations of Ca^{++}-ions for optimal activity of lyases	Remarks	Authors
Erwinia carotovora	10^{-3} - $4 \cdot 10^{-3}$M	after removal by dialysis	4*
E. carotovora	10^{-3}M		387
E. carotovora var. *atroseptica*	10^{-3}M		164
E. chrysanthemi	10^{-3}M		140
Bacillus polymyxa	0.5 - $2 \cdot 10^{-3}$M	higher concentrations progressively inhibitory	286 288
Pseudomonas sp.	10^{-3}M	10^{-4}M gave a two-fold increase	314
Clostridium multifermentans	0.5 - $2 \cdot 10^{-3}$M	higher concentrations progressively inhibitory	252 253 271
Arthrobacter 547	between 0.2 and $0.8 \cdot 10^{-3}$M	Figure 55	331
Pectinol R-10, commercial	below $5 \cdot 10^{-2}$M	Figure 51	7
Aspergillus carbonarius	0.13M		119 120
A. carbonarius	0.12M	activity increases from pH 5.2 to 8.5	3
Colletotrichum trifolii	10^{-3}M		172
Rhizoctonia solani	10^{-3}M	higher concentrations inhibitory	22
R. solani		10^{-3}M without effect but EDTA slightly inhibitory	345
Fusarium solani f.sp. *phaseoli*	1.2×10^{-2}M	gelling above 10^{-3}M	36
F. solani f.sp. *cucurbitae*	10^{-4}M	degraded pectin more rapidly than pectate in the presence of Ca^{++}	168
F. solani f.sp. *cucurbitae*	10^{-3}M	breakdown of pectate inhibited when the uronic acid/Ca^{++} ratio is ≤ 2	174

Table 17 continued

Organisms	Concentrations of Ca^{++}-ions for optimal activity of lyases	Remarks	Authors
F. solani f.sp. cucurbitae	10^{-3}M	this conc. gives about 5% stimulation	171
Proma betae	$1.7 \cdot 10^{-3}$M	10^{-8}-10^{-4}M without effect	73
Penicillium expansum	about $5 \cdot 10^{-2}$M	Figure 52	372

*Wood's investigations took place before the discovery of the lyases. However, the enzyme is without doubt a lyase.

Nagel & Vaughn (1961, 1962) found Na^{+}-, Mg^{++}-, Sr^{++}-, Ba^{++}-, Mn^{++}-, Zn^{++}-, Cu^{++}-, and Fe^{+++}-ions ineffective, whereas Preiss & Ashwell (1963) found 50% inhibition by 0.05M KCl, 0.07M NaCl, 0.02M K_2HPO_4, 0.02M K_2SO_4 and 90% inhibition by 10^{-4}M $MnCl_2$, and a further 40% stimulation by 10^{-4}M $MgCl_2$. MacMillan & Vaughn (1964) and MacMillan & Phaff (1966) stated that the stimulating effect decreases in the following order, Ca^{++}, Sr^{++}, Mn^{++}, Mg^{++}, and Ba^{++} and no stimulation by Zn^{++}. Rombouts (1972) found the same stimulating effect of Ca^{++} and Mg^{++}, whereas Sr^{++}, Ba^{++}, and Mn^{++} had little or no effect. $5 \cdot 10^{-2}$M Ca^{++} stimulated more than the same concentration of Mg^{++}. Mn^{++} was also stimulating, but had a maximum at $2.5 \cdot 10^{-2}$M (Spalding & Abdul-Baki 1973). Finally, Hancock (1976) found little effect of Na^{+}, K^{+}, Mg^{++}, while Li^{+} gave 30% inhibition of enzyme from culture.

In summary the results give the picture shown in Table 18.

Table 18. Effect of cations on the activity of lyases from various microorganisms as cited in the literature.

Organisms	Elements											Authors
	Li^{+}	Na^{+}	K^{+}	Mg^{++}	Ca^{++}	Sr^{++}	Ba^{++}	Mn^{++}	Zn^{++}	Cu^{++}	Fe^{+++}	
P. debaryanum				s								17*
B. polymyxa		0		0	s	0	0	0	0	0	0	286,288
Pseudomonas sp.		i	i	s	s			i				314
C. multifermentans				s	s	s	s	s	0			252,254
Arthrobacter 547				s	s	0	0	0				331
P. expansum				s	s			s				372
F. solani f.sp. cucurbitae	i	0	0	0	s							170

s = stimulating; i = inhibitory; 0 = no effect.

*Before the discovery of the lyases, see Figure 8 and text.

5.6. Summary of Previous Investigations on the Occurrence, Production and Properties of Pectolytic Enzymes and Toxic Substances Active During Pathogenesis

The effect of calcium on storage products such as the irradiated and non-irradiated carrots discussed above is important, but of equal importance is the processes that lead to this effect, which either develops in the plant tissue itself or is caused by enzymes during pathogenesis. A comprehensive knowledge of these processes is necessary in order to draw conclusions regarding future possibilities for use of calcium treatment as a protective measure in common practice. With this in mind, knowledge cannot be limited to studies of a small number of pathogens attacking a single product.

However, the following review should not be considered in this light only, but also as a background for and an introduction to the present author's investigations of pectolytic enzymes.

Several more or less extensive reviews with different aims have been published on various aspects of the subject. Therefore, only a short outline will be given here on the results that led up to the present knowledge.

Brown (1915) gave a historical review on the old literature on the physiology of parasitism; he mainly discussed the similarities between *Botrytis cinerea* Pers. ex Fr. and *Sclerotinia sclerotiorum* (Lib.) de By. and criticized the investigations on the phytotoxic effect of oxalic acid. In a review on the physiology of host-parasite relations, this author (Brown 1936) discussed the differences between the facultative and the obligate parasites regarding their host range and ways of attack. For the former group, he mentions enzymes and thermostable compounds together with several other compounds and mechanisms as the cause of wilting, whereas for the latter group there was no such list to present. In a comparison between *B. cinerea* and *Puccinia graminis* Pers., Brown mentions that the former killed the host tissue in advance of growth, while the latter did not do so until a late phase of attack. This should be compared with the results of Thatcher (1942), who found a considerable increase in permeability in the host tissue some distance ahead of the attacks of both fungi.

Matus (1948) gave a review on the very extensive old literature about the pectolytic enzymes and presented a list of the large number of organisms producing them. While de Bary (1886) was the first to prove that tissue disintegration was brought about by a substance with the nature of an enzyme, Bourquelot & Hérissey (1898) were - according to Matus 1948 - the first to detect the hydrolytic degradation of pectin by pectolytic enzymes. In the present connection it may also be of interest to mention that the pectinesterase (PE) has been known since 1840 when Frémy discovered its presence in carrots (Matus 1948).

Matus' (1948) list of pectinase-producing organisms presents several genera of bacteria, *Phycomycetes*, *Ascomycetes*, *Basidiomycetes*, and *Fungi imperfecti*, further some animals and higher plants. A comparison with the more recent literature, however, shows that some of the older findings were not reproduced in later experiments. Endo & Miura (1961) present another long list of pectolytic fungi including saprophytes as well as pathogens.

In a review on ecological adaptations (Brown & Wood 1953) the importance of enzymatic adaptation - including the adaptively produced pectolytic enzymes - is discussed.

Brown (1955) discusses the chemical factors in the physiology of parasitism and notes the risk of error by ignoring the metabolites of the parasites and by transferring the findings in vitro to the behaviour of the parasite on the plant. Although pectolytic enzymes are probably always present in organisms able to macerate plant tissues, he found no general parallelism between the pathogenicity and the capacity to produce pectolytic enzymes in vitro. As will be shown below, this opinion has somewhat changed, as in some cases there is good accordance between in vitro and in vivo tests, in others not. Even from saprophytes very active pectolytic preparations may be obtained.

Brown (1955) concentrates mainly on the conditions of the host tissue, where turgidity is of decisive importance, on the conditions affecting enzyme production, where the composition of the medium and the question of the adaptively produced enzymes are important, and on the differences between the pectolytic systems, where there is an outstanding difference between the organisms. For example, he notes that the enzyme of *B. cinerea* is more retarded by concentrations of, e.g., Mg^{++} than

are equally active preparations of *P. debaryanum* or *Phytophthora erythroseptica* Pethyb. The two latter organisms and various soft-rotting bacteria have alkaline optima, whereas the former has an acid optimum. The pectolytic enzyme of *B. cinerea* does not require Ca^{++}-ions for its function, whereas *Erwinia carotovora var. carotovora* does. When the middle lamellae are broken down and the tissue has just lost its coherence, the bacterium exhibited greater action on the cell wall proper than did *B. cinerea*. This led to a discussion of whether the pectolytic enzymes kill the cells, or whether this effect is caused by a toxin. Much evidence points in the direction of the enzymes, as it was, e.g., shown that when tissue losses coherence along the line of the middle lamellae, the walls break up into flakes and the percentage of cells capable of plasmolysis diminishes after a time and finally falls to zero.

This killing effect during pathogenesis was first observed by de Bary (1886) and has been a matter of discussion ever since. One of the most profound and inspiring contributions was that of Brown (1965), which mainly dealt with the interaction or proportionality between maceration and cell death. Brown drew attention to the complex nature of the primary cell wall and to the fact that until now the plasmalemma and the tonoplast are not found mixed up with substances of a polysaccharide nature and therefore, other things being equal, pectolytic enzymes should not be able to attack them. There is invariably a parallel between the rate of dying protoplasts and the maceration, though there are differences. This applies, e.g. to *B. cinerea*, where the maceration precedes the action on the protoplasts, whereas the reverse is the case with enzymes from *E. carotovora var. carotovora*. In this connection, Brown mentions the work of Tribe (1955), who cites the same killing effect on free protoplasts and in situ protoplasts, on the basis of a plasmolysis-deplasmolysis test. Based on these facts, Brown writes that an enzymic toxin active upon essential structural constituents would be the obvious suggestion.

Brown (1965) discussed the many reasonable reasons for discrepancies between in vitro and in vivo tests, and in this connection he mentions the differences in carbon sources in the media and the importance of the condition of the plant tissues, not the least the turgidity. Some pathogens are active inde-

pendent of the turgidity of the tissue, while others may only attack when the tissue is fully turgid. In such cases, even saprophytes may be able to attack.

The rate of action in the wound is important as the exposed tissue rapidly loses turgidity and starts suberization and formation of a new periderm. Therefore, based on the amounts of enzyme produced up to a fixed time, nothing can be predicted about the ability to attack the tissue. In vitro saprophytes may surpass the parasites in the production of pectolytic enzymes.

Wood (1960) calls attention to the constitution of the cell walls in his review on pectolytic and cellulolytic enzymes in plant diseases. He mentions that the middle lamellae of young plants mainly, if not solely, consist of pectic substances, while the secondary thickened cell walls are impregnated with lignin. The primary and secondary cell walls contain microfibrils surrounded by or imbedded in pectic substances and hemicelluloses in a matrix that is continuous with the middle lamellae (cf. Aspinall 1973, Keegstra et al. 1973, Bateman & Basham 1976). Some of the pectic substances are extractable with cold water and with diluted acids or alkalis, others are not. Besides the degree of esterification, the reasons for this may be cross-linkages between adjacent polygalacturonide chains through calcium, or protection from the imbedding in the above-mentioned matrix of the primary cell walls and middle lamellae - but there are other possibilities too. The fact that the de-esterification with PE is incomplete with some 10% of the methyl ester groups left unhydrolyzed under optimal conditions may have the same reason (Solms & Deuel 1955).

Wood (1960) discussed the production of pectolytic enzymes by plant pathogens in vitro and in vivo. Some pathogens only produce enzymes or some enzymes in the presence of inducers, whereas others readily produce them under a variety of conditions and without inducers. He assumed that production of pectolytic enzymes is proportional to the cell growth, but sometimes enzyme activity continues to increase after the active growth has stopped. Further, this author discussed the importance of working under specified conditions, or of getting rid of contaminating enzymes. He states that surveys of large groups of pathogens to find their ability to secrete pectolytic enzymes

are of limited value, because of the variation in behaviour between isolates of the same species. The present author disagrees with this statement and, in view of the vast literature on the subject, most other authors do so too.

In order to be sure that pectolytic enzymes play a part in the development of a disease, it is necessary to prove their presence in diseased tissue in significant quantities and with properties different from those of the plant. However, the indigenous enzymes usually occur in small amounts compared to those of the pathogen (cf. Bateman & Millar (1966) and the review on pectolytic enzymes in higher plants, paragraph 9.1.3).

It is often a matter of discussion at which state of the development of the disease the pectolytic enzymes have most influence. Their importance is clear when the disease has been initiated, but there are indications showing that they may play a role from the very start. For example, the wound parasite *Penicillium italicum* Wehmer may readily invade undamaged citrus fruit tissue when pectic substances are present, as is always the case in exposed wound tissues, and once initiated the production and action of the pectolytic enzymes is a self-accelerating process. In this connection, differences have been noted between soft-rot, leaf-spot, vascular wilt, and obligate parasitism, in all of which the enzymes occur (cf. e.g. Cole & Wood 1961a, b, Hancock & Millar 1965). This supplements the review of Husain & Kelman (1959) on the same and other diseases. It is evident that many pathogenic fungi and bacteria produce pectolytic enzymes, and many papers indicate a correlation between this ability and the pathogenicity or virulence, while in other cases no such indication was found. Therefore this property does not explain why the organism is pathogenic. Rather, these enzymes act in collaboration with other compounds in order to make an organism pathogenic (Bateman & Millar 1966). Such other compounds may be other enzymes or toxins produced by the parasites, and inhibitors or modifiers in the plant tissue. Although mechanisms that specifically regulate the activity of pectolytic enzymes in the plant tissue are unknown, they are supposed to be present in cases of symbiosis where one of the organisms produces pectin-degrading enzymes (cf. Bateman & Millar 1966, Nutman 1965). In other cases there may be changes in the pectolytic enzymes of host origin during pathogenesis and interaction with

those produced by pathogens, and there is evidence that proteases and other non-pectolytic enzymes may act synergistically with the pectolytic enzymes of the same pathogen. Obviously, the cellulases do not have such an effect nor do they seem to contribute to the maceration. Metabolites such as oxalic acid may also interact with the enzymes, as they - like chelating agents - may remove the calcium from the tissue and there enhance the activity of the polygalacturonases and reduce that of the lyases.

In a review on the biochemistry of the degradation of cell wall compounds, Albersheim, Jones & English (1969) set forth the hypothesis that the interaction between the pathogen and the carbohydrates of the host, which determines the ability of the pathogen to produce cell-wall-degrading enzymes, is necessary for the initiation of a disease. The hypothesis is supported by a series of examples from the literature. At the same time, these authors show that across the biological spectrum, cell surfaces are intimately involved in pathogenesis and constitute sites for the effect of carbohydrates and for the initiation of the infective process. The conditions at these sites vary considerably, for which reason the synthesis of cell-wall-degrading enzymes may be induced or repressed. This makes it an advantage for the pathogens to have a series of enzymes with different properties as this increases the possibilities to initiate an attack.

Rombouts & Pilnik (1972) compiled a survey on - as they say - the depolymerases (polygalacturonases and lyases) which have been adequately purified from accompanying pectolytic enzymes and sufficiently studied to be placed in one of the eight groups defined in recent biochemistry. This review deals to some extent with other organisms and presents details other than those given by the present author. For example, Rombouts & Pilnik give the temperature characteristics of the enzymes and the degree of hydrolysis or depolymerization in general, in relation to the degree of esterification or to the loss in viscosity of the pectic substances in solution.

According to Ginzburg (1961), there is some evidence for the occurrence of a protein gel cross-linked with cations, e.g. Ca^{++}, in the middle lamellae. This is questionable, however, but as the distinction between the middle lamellae and the primary

cell wall is not very well defined, implications are not unlikely (Bateman & Basham 1976). In recent years it has been clearly stated that, besides cellulose, hemicellulose, and pectic substances, the primary cell walls contain proteins and possibly lignin too; further that the carbohydrate polymers of the plant cell walls are much more complex than hitherto assumed (Albersheim et al. 1973, Aspinall 1973, Keegstra et al. 1973, Lamport 1973, Wheeler 1975, Bateman & Basham 1976). Therefore it is not surprising that several enzymes other than the pectolytic ones may contribute to cell wall disintegration during pathogenesis, though not only as purified enzymes (Wheeler 1975, Bateman & Basham 1976).

6. PREVIOUS INVESTIGATIONS WITH *SCLEROTINIA SCLEROTIORUM* AND *BOTRYTIS CINEREA*

Sclerotinia sclerotiorum (Lib.) de By. and *Botrytis cinerea* Pers. ex Fr. were used in almost all the experiments presented below, for which reason a detailed review of previous investigations concerning the pectolytic activity of these fungi is given here as an introduction to the presentation of the present author's experiments.

6.1. *Sclerotinia sclerotiorum*

It is often a difficult task how to distinguish between closely related species, as it is a matter of subjective impression which characters are of decisive importance for the separation, or how many characters should differ before there is a separate species. If, on one hand, the species are lumped together in too broad taxa, it will be necessary to distinguish between their special characters in other ways; on the other hand, if the species are separated on the basis of a series of "characters of minor importance", one may obtain species of academic interest only.

In applied plant pathology, species of *Sclerotinia* are often separated on the basis of sclerotial size or host species. This is, of course, too rough a method and consequently Purdy (1955) compared these characters with the apothecia, asci and ascospores normally regarded as decisive characters. The comparisons did not result in any significant differences, so Purdy combined several species that only differ in sclerotial size and host specificity under *Sclerotinia sclerotiorum*.

Korf & Dumont (1972) proposed a new genus, *Whetzeliana*, for *Sclerotinia* species producing free sclerotia not incorporating host tissues (*S. sclerotiorum*, *S. tuberosa* (Fedw. ex Mérat) Fckl.). Buchwald & Neergaard (1972) made a plea for retention of the broader sensu of the genus with *S. sclerotiorum* as type species. Korf (1974) was opposed to their arguments.

Later, Wong & Willetts (1975a, b) studied the mycelial interactions and electrophoretic separation of proteins and several enzymes, and compared the results to the size of sclerotia. They were thus able to distinguish clearly between *S. minor* Jagger, *S. trifoliorum* Eriks., *S. sclerotiorum* and *S. tuberosa*.

The present author agrees with Purdy (1955) about the importance of the size of the asci and ascospores, as also with Korf & Dumont (1972), that this feature is not always enough to separate species, such as also stated by Wong & Willetts (1975a, b); neither - at least above species level - is the question of whether the sclerotia are intermingled with the host tissue or not. However, if all facts - including the ancienity - are considered, it is both correct and practical to keep the species separate with *S. sclerotiorum* as the type. Consequently this has been done by Mordue & Holliday (1976), who retain the name *Sclerotinia sclerotiorum* without discussion in the CMI descriptions.

Already de Bary (1886) found that *S. sclerotiorum* gave rise to disorder in plant tissues, e.g. the storage roots of carrots, some distance ahead of the hyphae, which principally grew intercellularly in the tissues. de Bary also found that a toxic substance leaked out of the fungal cells during growth, and he supposed that it penetrated the plant cell walls, disturbed the cell membranes and caused death of the cells, because a number of cells in the invaded tissues were unable to plasmolyse. Using the filtered juice from, e.g., attacked carrots, de Bary (1886) showed that it could cause serious destruction of healthy tissues within 2-3 hours. This specific effect could be removed by boiling the juice. As a result of these facts he proposed that the disorder of the tissue and the cell separation were caused by a ferment or an enzyme produced by the fungus.

Further, de Bary (1886) found that the hyphae of *S. sclerotiorum* exudated oxalic acid into the cell juice, where he found up to 0.3% as oxalates (mainly the potassium salt). Older hyphae were seen incrusted with Ca-oxalate. In spite of these facts, de Bary was unable to demonstrate any effect of oxalic acid, for which reason his final proposal was that the effect remaining after boiling was a quantitative one rather than one caused by the oxalates present.

Surprisingly, it was more than half a century before studies of the enzymes and/or toxic substances of *S. sclerotiorum* progressed any further.

Overall (1952) could not demonstrate the presence of toxic substances produced by *S. sclerotiorum* until the mycelium reached its maximal growth; hereafter a thermostable toxin developed in the medium. Paper chromatographic examinations re-

vealed the presence of oxalic acid in ageing cultures. Tests with increasing concentrations of this compound at various pH levels showed that the effect decreased with increasing pH, being very low at pH 5 or higher levels, and that concentrations of the order of that found in the diseased tissue were sufficient to cause the leakage described at pH 3.9. On this basis, Overell (1952) suggested that the undissociated molecule and the monobasic ion are the active toxic forms of oxalic acid, and that it may be the most important and perhaps the sole toxic matter produced by *S. sclerotiorum*.

Echandi & Walker (1957) found very little production of oxalic acid in a 10-day-old wheat bran culture of *S. sclerotiorum*.

Maxwell & Lumsden (1970) went further and found the production of oxalic acid to depend on the medium as well as on the isolate of *S. sclerotiorum* because 17 isolates tested under uniform conditions produced from 1.4 to 78.3 mM. One of the isolates was inoculated on bean hypocotyls, where 48.3 mg oxalic acid per gramme dry weight of host tissue was found after 4 days' incubation. In contrast to Overell (1952) Maxwell & Lumsden (1970) found the production of oxalic acid proportional to the growth of the fungus and to the disease index on bean hypocotyls. At the same time, the pH dropped to 4.5 where, according to Overell (1952), the toxic effect of oxalic acid is rather high. The decrease in pH started at the edge of the lesions, but the lesions were not caused by the low pH; it was more likely that they were caused by the activity of the pectolytic enzymes that is enhanced at the low pH - or possibly by oxalic acid - confer Hancock (1966a), who stated that the production of oxalic acid is great enough to kill the plant tissues.

Maxwell (1973) extracted from the hyphae of *S. sclerotiorum* (*Whetzeliana sclerotiorum*) an enzyme that catalyzes the formation of oxalic acid and acetate from oxaloacetate, which may originate from a transamination of aspartate. This should be compared with the common use of aspartate as a nitrogen source in the growth media, which point is further discussed under *Botrytis cinerea* and in the work of the present author. Though apparently not dependent on $MgCl_2$, the production of oxalic acid is much enhanced in its presence.

Vega, Corsini & le Tourneau (1970) studied the production

of non-volatile organic acids by *S. sclerotiorum* and found an accumulation of oxalic, succinic, malic, fumaric, and glycolic acids in the medium. After 2 weeks' growth on the media, the pH reached a minimum at 3.4 with the content of dicarboxylic acids at a maximum. The total amount of these acids decreased and the amount of glycolic acid increased with longer incubation. The present author supposes that this means that the dicarboxylic acids were slowly metabolized.

By increasing the pH of the medium, a greater amount of oxalic acid was produced. Further, the amount and relative proportions of the acids depended on the medium used; on a mannitol-salt medium, for example, the amount of oxalic acid and glycolic acid increased slowly during the whole growth period.

Echandi & Walker (1957) were the first to take up the study of the pectolytic enzymes produced by *S. sclerotiorum* after de Bary (1886). They demonstrated the production of pectinesterase (PE) as well as of a polygalacturonase (PG) equally active on 9% methylated pectin and on Na-polypectate. With reference to this result, it should not be ignored that the presence of PE in the reaction mixture may have demethylated the pectin fast enough for the PG to affect it as if it was pectic acid. Both enzymes are very active at the pH used. It is doubtful whether this exhausts the possibilities as it was later shown that the fungus degrades pectin in the absence of PE and that it produces a polymethylgalacturonase (PMG) (Endo 1961a, Endo & Miura 1961).

Further, Echandi & Walker (1957) showed that the macerating activity of the culture filtrate was inhibited in the presence of juice from susceptible (carrot roots, radish roots, and cucumber fruits) as well as from resistant (potato tubers and onion bulbs) storage organs.

Using sunflowers (*Helianthus annuus* L.) for experiments, Hancock (1966a) showed that the PG and the PE activity from *S. sclerotiorum* caused a loss of pectic acid and a reduction of the total content of methoxyl groups, respectively, in the attacked tissue. Only now and then could a weak polygalacturonate-transeliminase (i.e. pectate lyase) activity be detected in the attacked tissue when tested at pH 9.

During the attack, the pH dropped from 6.4 to 4.5, which is in favour of the PG and PE activity. At the same time no PG activity could be detected in the healthy tissue, while PE ac-

tivity was demonstrated when NaCl was added. Finally, Hancock (1966a) stated that the addition of Ca^{++}-ions greatly inhibited maceration of the tissue.

Enzymes of *S. sclerotiorum* also affect the hemicellulose and the cellulose components of the cell walls, consequently these enzymes are thought to play a role in the deterioration of the tissues too, but commonly to a lesser extent than the pectolytic enzymes (Hancock 1967, Lumsden 1969). However, it has been shown that the activity of cellulase may be proportional to the progress of the disease (cf. Bateman 1964). These enzymes seem not affected by calcium.

van den Berg & Lentz (1968) stated that growth and survival of *S. sclerotiorum* and *B. cinerea* depend on the relative humidity, the temperature and on the type and strain of organism. Without nutrients, survival varied from 12 months at 95-100% rh and 0°C to less than 1 month below 95% rh and at 20°C. Årsvoll's (1969) observations agree with this. In the presence of nutrients, growth occurred at 93% as the limiting equilibrium relative humidity. Below this there was no growth and the mycelium did not survive for more than 1 month. The two organisms showed equal growth from -0.8°C to 35°C with an optimal temperature at 20°C. On the basis of these facts, van den Berg & Yang (1969) studied the effect of the two organisms on carrots at 90-100% r.h. The authors were unable to detect significant amounts of lyase from growth on the surface of the carrots, for which reason they concluded that the pectolytic activity of both fungi under these conditions is limited to PE, endo-PG, and possibly to endo-PMG (cf. Bateman & Millar 1966).

Morrall, Duczek & Sheard (1972) compared on turnips and carrots 38 isolates of *S. sclerotiorum* from 23 hosts with enzymes produced on a defined medium. They found the most important enzyme to be endo-PG. Some of the isolates also produced exo-PG and PE, but many isolates produced little or none of the latter. Lyase activity was never detected. These authors did not find any agreement between pathogenity and enzyme activity, wherefore they concluded that the enzymes were not the agent chiefly responsible for the pathogenicity. In spite of all the differences between the 38 isolates they could not be placed in groups with related characteristics.

Lumsden (1976) came to a rather different result in his

studies on the production of endo-PG, exo-PG and PE from *S. sclerotiorum* both in diseased tissue early in pathogenesis and in culture. The production of endo-PG in culture was suppressed by glucose. Bean PE and fungal PE were distinguished by the fact that the former was only active in the presence of 0.1M NaCl or $CaCl_2$, whereas the latter was not affected by salts. A correlation was observed between the virulence of various *S. sclerotiorum* isolates and the PG-activity but not with the PE-activity.

Thatcher (1939, 1942) found increased permeability in the tissue in advance of the growth. This is discussed in further detail under *B. cinerea* below.

6.2. *Botrytis cinerea*

B. cinerea has been one of the main objects for studies of the physiology of parasitism, which has been extensively discussed in the reviews of Brown (1915, 1936, 1955, 1965). In his first paper, it was stated that *B. cinerea* and *S. sclerotiorum* are closely allied and show many similarities in their mode of parasitism. On this basis much labour and much literature have been expended on discussing whether the papers appearing after de Bary (1886) are in agreement with de Bary's results, and whether oxalic acid is produced and if it is of any primary significance - although these papers deal with *B. cinerea* and not with de Bary's *S. sclerotiorum*.

According to Brown (1915), Kissling, Ward and Nordhausen all found the cell-wall-dissolving enzymes, but they could not demonstrate that *B. cinerea* produced any toxin, though Nordhausen added that it is not unlikely that oxalic acid may play a role under some circumstances.

Smith (1902) compared small concentrations of oxalic acid with extracts of *B. cinerea* and concluded that oxalic acid could be the compound responsible for the effect. Brown (1915, 1936) opposed this idea and criticized Smith's method, not least because he found that other acids have the same effect. Brown (1915) supported his criticism by experimental results stating that the macerating ability of *B. cinerea* extracts was lost at 65°C, was reduced significantly at 40°C in one hour, and at 50°C in 15 minutes the activity was reduced to less than the half. Further, he found two principles, one diffusible and one

not diffusible, by dialysis, and he obtained convincing proof that soluble oxalates played no part whatsoever in the lethal activity of the extract. This is in agreement with de Bary (1886).

The studies and discussions on the role of enzymes and toxins, or both, in the maceration and killing effect continued. Gentile (1951) studied the toxin produced by *B. cinerea* and showed it to be a thermostable, non-volatile compound soluble in alcohol and acetone and able to wilt tomato seedlings. He isolated several compounds produced by the fungus, but had to state that none of them could be responsible for the toxic effect. Surprisingly enough he did not deal with the question of oxalic acid, and, in contrast to others, he related the toxin production to rapid growth, utilization of carbohydrates and lowering the pH value. Later, he dealt with the problem of carbohydrate metabolism and oxalic acid synthesis of *B. cinerea* (Gentile 1954). Gentile's results showed that the pH increased during growth and the oxalic acid formation followed the rapid utilization of glucose, but it did not accompany the growth of the fungus as most of the oxalic acid was produced before the exponential growth, i.e. in the first week of growth. The present author's results are not in agreement with these results. Later, the production of oxalic acid levelled out and the amount started to decrease. Further, Gentile (1954) showed that *B. cinerea* converted malic, oxalosuccinic, oxaloacetic and succinic acids to oxalic acid in order of decreasing rate. The oxalic acid accumulated because it was not further utilized. These results are in agreement with those of de Stevens, DeBaun & Nord (1951), who showed the pathways for oxalate formation and noted physiological alkalinity as a primary factor for the accumulation of oxalic acid. This leaves the question of whether the oxalic acid in the cultures of *B. cinerea* and *S. sclerotiorum* in the present author's experiments could be a result of the relatively high amounts of aspartate in the growth medium used, as one of the pathways is aspartate → oxaloacetic acid → oxalic acid. This pathway was definitely established by Maxwell (1973).

Thatcher (1939) studied the physical effects of some plant parasites using *Uromyces fabae* (Pers.) de By., *U. caryophyllinus* Wint., *S. sclerotiorum*, and *B. cinerea*. All the fungi had a considerably higher osmotic pressure in the cells than the particular hosts had in their cells. For *S. sclerotiorum* and *B. ci-*

nerea, he presents the following figures together with that of the celery tissue used as host:

Osmotic pressure in atmospheres:

S. sclerotiorum hyphae *Apium graveolens* L., petiole	23.5 9.4-17.4
B. cinerea hyphae *A. graveolens*, petiole	29.8 8.3

With a higher osmotic pressure, the fungi are able to remove water from neighbouring parenchyma. However, Djacenko (1971) showed that *S. sclerotiorum* may increase the osmotic potential in the hyphae up to a certain level parallel to the increase in carrot tissue (Figure 9). This should be seen in connection with the fact that this fungus causes the most severe attack on carrots with a low osmotic potential where the difference to that of the fungal cells is greatest. *B. cinerea* causes the most severe attack in carrots with a high osmotic potential (cf. e.g. Årsvoll 1969, Djacenko 1971, Goodliffe & Heale 1977, and Table 12 in the present paper).

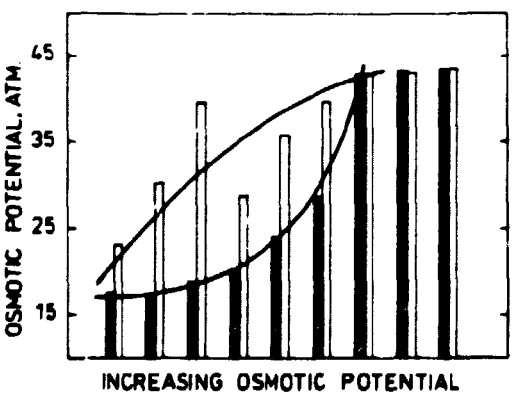

Figure 9. The osmotic potential of uninfected carrot-root-tissue (hatched columns) and of hyphae of *Sclerotinia* developing in the same root (white columns). The osmotic potential of the hyphae increases with increasing osmotic potential of the root tissue until a certain level. (Drawn after Djacenko 1971 Table 3).

In all cases the fungi increased the permeability of the host tissue in advance of the attack. The figures for the permeability at different distances from cells killed (necrotic tissue) by *S. sclerotiorum* and *B. cinerea* are set up in curves (Figure 10). In another series of experiments, Thatcher (1942) showed that the average deplasmolysis time for healthy cortical parenchyma of celery petioles and for those attacked by either *B. cinerea* or *S. sclerotiorum* (within one inch from discoloured zones) was 474, 102 and 99 seconds, respectively. Thatcher (1939) suggests that these destructive parasites have this effect on the basis of their pectin-digesting enzymes, and that both the permeability increase and the killing of cells contribute to satisfy their water and food requirements, but at the same time the water economy and water transport in infected plants are disturbed (cf. Dimond 1972).

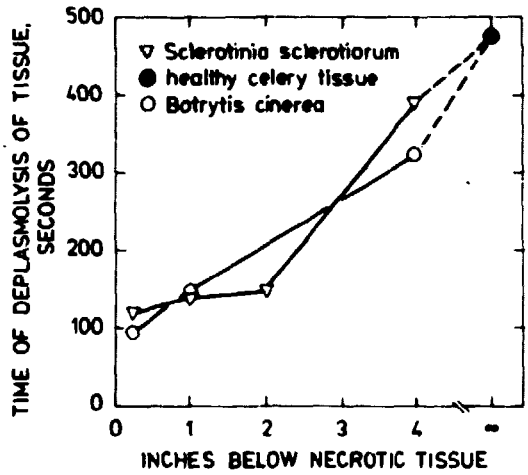

Figure 10. Water permeability of celery tissue as affected by *Botrytis cinerea* and *Sclerotinia sclerotiorum*. The permeability increases strongly from the healthy to the attacked tissue. (Drawn after Thatcher 1939 Table 6).

Hancock (1972) found the influx and efflux of water and urea and of electrolytic leakage less for sunflower tissue above lesions caused by *S. sclerotiorum* than for tissues of healthy hypocotyls. This is somewhat different from earlier investigations, consequently he was of the opinion that knowledge of the

nature of permeability changes in plants attacked by *S. sclerotiorum* needs revision. On the other hand, Hancock and his colleagues (Lai, Weinhold & Hancock 1968) found close agreement between the PG protein fraction and the fraction that caused the largest leakage of electrolytes from bean hypocotyls attacked by *R. solani*. The increased permeability was detectable prior to the development of symptoms.

The lower permeability to water as discussed above should be compared with an analogous effect observed after exposure to 250-500 rad α-rays (Stadelmann 1969).

Using the ruthenium red staining technique, pectin hydrolysis could not be determined in tissues more than two cell diameters away from disintegrating cells in a transverse direction, and not more than six to eight cell lengths away from such necrotic tissue in a longitudinal direction.

Thatcher (1939, 1942) also observed these phenomena caused by several other pathogens including obligate parasites as well as dry-rot organisms.

The roles of permeability in pathogenesis are further discussed in the review of Wheeler & Henshey (1968).

Tribe (1955) found that culture filtrates of *B. cinerea* and *E. carotovora var. carotovora* reduced the viscosity of pectate solutions, macerated parenchymatic tissue, and killed the cells in the macerated tissue. The filtrate from *B. cinerea* was active from pH 3.5 to 8.0, but from 6.0 to 8.0 it decreased rapidly. In contrast, *E. carotovora var. carotovora* was most active above pH 8.0 and reduced progressively to pH 5.5. By the neutral red method Tribe further showed that plasmalysing concentrations of salts or non-electrolytes retarded the killing effect, and he observed a thermostable toxic compound, but he did not identify it and neither does he mention oxalic acid.

Fernando & Stevenson (1952) observed both a quantitative and a qualitative difference between saprophytes and parasites, as there was a correlation between the capacity of attack and the amount of 'pectinase' exudated, and because their attacks were differently affected by the turgescence of the tissue. *Bacillus subtilis* Cohn and *B. megaterium* de By., which are normally termed saprophytes, were able to parasitise potato tissue injected with water. The same applies to *B. cinerea*, which is not a "normal" pathogen on potato tubers, but if the normal, sub-

turgid potato tissue is injected with water to a 4% increase *B. cinerea* enzymes are able to rot the tissue. At the same time it was shown that enzymes of *E. carotovora* are independent of the turgidity of the potato tissue. These results were confirmed by Brown (1955) who found the same behaviour of *P. debaryanum* as of *E. carotovora*.

Mishra (1953) determined the enzyme activity after growth of *B. cinerea* in a glucose-asparagin-salts medium (cf. Fernando 1937, Fernando & Stevenson 1952, Ashour 1954). As substrate for the enzyme activity, he used slices of potato tubers and compared them with inoculating the fungus itself onto potato tubers. His results agree with those of Fernando & Stevenson (1952), but he added that *B. cinerea* produces pectic enzymes in the potato tissue and stated that the attack was restricted because the enzymes could not diffuse into the normal tissue.

A few agents have the ability of separating the plant cells or rotting the tissues (Brown 1955). They almost exclusively are high alkalinity, strong acidity, and pectin-dissolving enzymes, but the causal connections are more complex than that. For example, *B. cinerea* and *Pythium debaryanum* grow on the same media, but it can be difficult to detect traces of pectolytic activity in culture filtrates from the latter, while from the former a strong activity is normally found. When the fungi are inoculated on wounded potatoes the situation is the opposite. Here *P. debaryanum* rapidly set in with a fast-developing soft-rot that destroys the potatoes, while even a very high inoculum with *B. cinerea* spores, together with extra nutrients, only gives a weak attack that soon dries out. Brown (1955) gives other examples too. Consequently it is also a question of the ability to attack living tissue and of the conditions of that tissue (Brown 1936, 1955). Other parameters must be involved, however, as this behaviour of *B. cinerea* is somewhat opposite to that found with carrots in several other cases, including the work of the present author as discussed in paragraph 2.2.1.2. above.

In relation to the present work it is important to note (Brown 1955) that the macerating enzyme from *E. carotovora var. carotovora* requires the presence of Ca^{++}-ions, while that of *B. cinerea* does not.

Several other authors have studied the activity of the pec-

tolytic enzymes of *B. cinerea* either alone or in comparison with those of other organisms. Thus, Fernando (1937) compared the 'pectinase' activity of *B. cinerea* with that of *P. debaryanum*, *E. carotovora*, and *Bacillus subtilis*. Using Brown's method (1915) for a study of maceration, he found that *B. cinerea* showed an optimum activity on the acid side and an optimum for the three others on the alkaline side of the pH scale. Further, Fernando (1937) studied the effect of several organic and inorganic compounds on the production of pectolytic enzymes, and he concluded that asparagin, peptone, and ammonium tartrate caused a drift in the pH value to 6-7 and a production of very active enzyme solutions. In contrast, the ammonium salts of inorganic anions caused the pH value to drift to about 3, and a very low enzyme activity resulted. The results indicated that when the pH value decreased to about 5.5 or lower, the enzyme exudation was reduced. These rules seem to apply to both *B. cinerea* and *E. carotovora*. This shows one kind of substrate dependence, but there are others.

Cole (1956) cited Proskuriakov & Ossipov (1939) as the first to state that PG and PE are at least partly adaptive enzymes in *B. cinerea*. Gäumann & Böhni (1947a) studied this phenomenon more closely and found a different behaviour of pectinase, i.e. polygalacturonase (PG), and pectase, i.e. pectinesterase (PE). On glucose, there was a rapid production of PG while in the presence of pectin there was a delay or lag phase before the rapid production of PG set in. The latter was also the case for the production of PE, while this enzyme was not produced at all with glucose as carbon source. On this basis Gäumann & Böhni (1947a) concluded that in *B. cinerea* PG and PE are constitutive and adaptive enzymes, respectively, as is also the case for the enzymes produced by *A. niger* (Gäumann & Böhni 1947b). In the pectin-containing medium, the pH drifts down to below 3 in the first 5 days, followed by an increase to above 7 after 4 weeks. This is in close agreement with the present author's results. On glucose, the pH reached 7 in about 11-14 days. These facts should also be taken into account when stating anything about enzyme production. The formation of 'pectinase' may be induced in *B. cinerea* on a glucose asparagin medium, but the production depends on their proportions (Brown & Wood 1953). Also Ashour (1954) studied the growth and enzyme production of *B. cinerea*

and found an increase with increasing amounts of asparagin in the medium. Sucrose and glucose gave a reduced growth and enzyme production. The interaction between these compounds showed that mycelial growth and enzyme activity were inversely correlated with the C/N ratio; when reducing the ratio by increasing the amount of asparagin the correlation became direct. The presence of pectin favoured the production of PG and an increased concentration of $MgSO_4$ reduced both the production and the activity of the enzyme at the optimal pH 5 as noted above (cf. Figure 8).

Winstead & Walker (1954) compared the pectolytic activity of several organisms. In contrast to the *Fusarium* species and *Pseudomonas solanacearum* (E.F. Sm.) E.F. Sm., *B. cinerea*, and *Alternaria solani* Sorauer did not produce PE in replacement cultures. On the other hand, *B. cinerea* had a much higher enzyme activity than the other organisms when grown on wheat bran and with almost equal amounts of PE and PG.

Extracts from diseased and fresh plant tissues and from culture filtrates of growth on artificial media were assayed for pectolytic activity on plant tissue and on soluble pectic substances (lemon pectin with 12.3% methoxyl, apple pectin with 9.7% methoxyl, Na-NH_4-pectate with 4.2% Na^+ and 1.3% NH_4^+) by Cole (1956). Maceration was tested by Brown's method (1915) with the disks of tissue immersed in McIlvaine's buffer at the required pH, and the toxicity was determined by the neutral red method described by Tribe (1955). Though *B. cinerea* causes the death of the protoplasts and rotting of the apple tissue, little or no pectolytic activity was detected in the extracts of the rotted tissue. This fact may be a result of interaction between the pectolytic enzymes and the oxidation system, as found probable both by Cole (1956) and by Cole & Wood (1961b), who found the inhibitory compound to be leucoanthocyanins changed to other compounds by polyphenoloxidase.

Wood & Gupta (1958) compared the pectolytic enzymes produced by *B. cinerea*, *P. debaryanum* and *E. carotovora var. carotovora* measuring their effects on 1% solutions of high methoxyl pectin (10.4% methoxyl corresponding to 71% esterification), Na-polypectate, Na-pectate from de-esterified pectin, or of Na-pectate from neutralized pectic acid. The organisms were grown on glucose-asparagin-salts media adapted to suit special requirements. Cell-free filtrates were prepared from growth on re-

spective media by centrifugation and storing under toluene, and then used for the reactions. Protopectinase activity, i.e. the macerating effect, was tested on turgid potato tissue by Brown's method (1915), PE activity was measured by titration as the rate of liberation of carboxyl groups from the pectin solutions, and PG activity was measured as the increase of reducing groups in the reaction mixture and as the overall decomposition of the molecule chain expressed as viscosity loss. The present author has redrawn the figures given by Wood & Gupta (1958) in order to facilitate the discussion of similarities and dissimilarities between the three organisms (Figure 11). They all exhibited a high viscosity-reducing activity, though somewhat lower for *P. debaryanum*. On Na-polypectate, *B. cinerea* and *E. carotovora var. carotovora* were very active while *P. debaryanum* was practically inactive. On Na-pectate (de-esterified), *B. cinerea* was very active, *E. carotovora var. carotovora* somewhat less active, and *P. debaryanum* inactive. On Na-pectate (neutralized pectic acid), *E. carotovora var. carotovora* was very active, *B. cinerea* clearly less active, and *P. debaryanum* inactive. It was further shown that the activity of *P. debaryanum* decreased with the decrease in methoxyl content.

Wood & Gupta (1958) also found the three organisms clearly different in their PG activity. *P. debaryanum* showed very little, *E. carotovora var. carotovora* moderate, and *B. cinerea* high activity. The greatest difference between *B. cinerea* and *E. carotovora var. carotovora* was on pectin where the latter had its lowest activity. It was finally shown by chromatography that *B. cinerea* liberated galacturonic acid without detectable amounts of intermediate compounds. *E. carotovora var. carotovora* produced only intermediate compounds from any of the three substrates.

Extracts from apples rotted by *Sclerotinia fructigena* Aderh. & Ruhl. (rapidly developing firm rot), *Potebniamyces discolor* (Mouton & Sacc.) Smerlis (slowly developing firm to soft rot), *Penicillium expansum* Link emend. Thom (slowly developing soft rot), and *B. cinerea* (rapidly developing soft rot) were compared by Cole & Wood (1961a, b) after centrifugation and adjusting the pH to 5.0, and by using the methods described by Cole (1956) and Wood & Gupta (1958). All the fungi had a high PE activity. *S. fructigena* and *B. cinerea* exhibited little or no PG and macerating activity, while these enzymes were present in extracts from

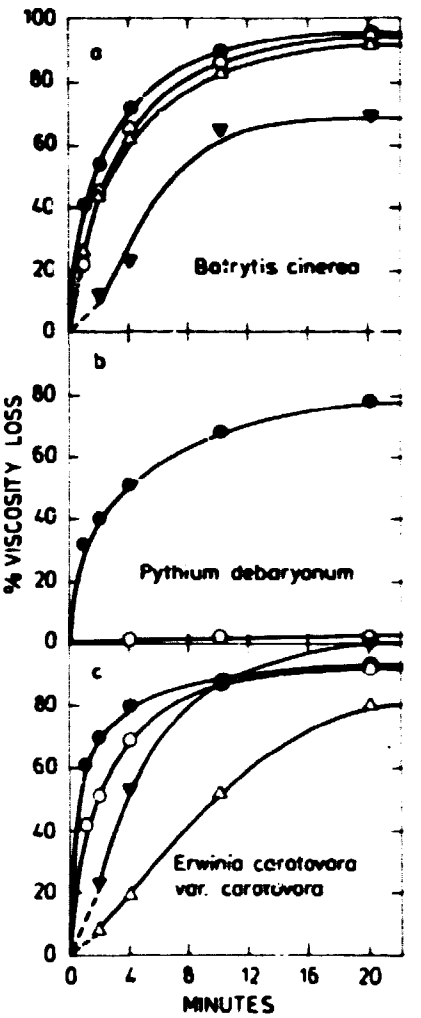

Figure 11. The degradative effect of culture filtrates from *Botrytis cinerea*, *Pythium debaryanum*, and *Erwinia carotovora var. carotovora* on pectic substances as measured by the rate of viscosity loss. ● : pectin with a high methoxyl content; ○ : Na-polypectate; △ : Na-pectate, pectin de-esterified by pectinesterase from the plant; ▼ : Na-pectate, neutralized pectic acid. *P. debaryanum* had no effect on the two Na-pectates. (Drawn after Wood & Gupta 1958 Table 1).

the *P. expansum* attacks. All three fungi produced PG in liquid media. These differences could be due to susceptibility or re-

sistance to the effect of the oxidizing systems in the apples (compare above). In the cases with attacks by *S. fructigena* or *B. cinerea*, most of the activity remained on the apple fibres, while that of *P. expansum* leached out into the extract. This may be another reason for the differences. During the attack and on digests of polypectate and pectin, *P. expansum* produced large amounts of galacturonic acid and short chain polymers of this acid. *P. discolor* degraded the pectic substances more slowly than did the other fungi.

In this connection it should be added that *B. cinerea* enzyme preparations were second to those of *Coniella diplodiella* (Speg.) Petr. & Syd. but much better than preparations from many other fungi for clarifying apple juice. In this case it was shown that *B. cinerea* produces PMG, PG and PE (Endo 1961a, Endo & Miura 1961).

B. cinerea is much less pathogenic to beans (*Vicia faba* L.) than is *B. faba* Sardiña, but as the former fungus produces considerably more PG and PE than the latter, it is unlikely that the pectolytic enzymes play a primary role in this connection (Deverall & Wood 1961a, b). Here it is worth mentioning that the authors found calcium-deficient bean plants more susceptible than plants which had received normal amounts of nutrients. These facts were discussed by Deverall & Wood (1961a, b), who believed calcium to play an important role in the structure of the cell wall by its ability to form linkages between adjacent chains of pectic acid or not fully esterified pectins. The rigidity of the gel increases with the frequency of such linkages. It is, however, also possible that calcium-deficient cell walls are more permeable to substances that stimulate the germination and growth of pathogens.

It is clear that it is necessary to be cautious with results based only on enzymes produced in vitro and on their activity exhibited on more or less purified pectic substances. These problems were, for example, studied by Hancock, Miliar & Lorbeer (1964) when they compared the pectolytic and cellulolytic activities of *B. allii* Munn, *B. cinerea*, and *B. squamosa* Walker in vitro and in vivo after growth on potato dextrose broth (PDB), on detached onion leaves (*Allium cepa* L.) or on intact leaves on living plants. At the same time they distinguished between endo-PG and exo-PG. The results are summarized in the following outline:

Species	In PDB		In detached leaves		In intact leaves	
	endo-PG	exo-PG	endo-PG	exo-PG	endo-PG	exo-PG
B. allii	traces	-	+	+	+	+
B. cinerea	+	-	+	+	+	traces
B. squamosa	+	-	+	+	+	-

In other sets of conditions there may be a considerable production of exo-PG as measured by the amount of galacturonic acid (compare Wood & Gupta 1958, Kaji, Tagawa & Yamashita 1966, and the present author's work). The fungi were allowed to maintain a prolonged saprophytic existence in the necrotic tissue of the detached leaves, which the authors supposed to be conductive to the production of exo-PG. Further, it was supposed possible that products resulting from parasitic activity in the leaf tissues may act to induce exo-PG production during saprogenesis. All three species produce PE and cellulase under the three sets of conditions.

Kaji, Tagawa & Yamashita (1966) studied the relation between the pectolytic enzymes produced by *B. cinerea* and the maceration of plant tissue. The fungus was grown on a pectin-peptone-salts medium and crude enzyme solutions were prepared by salting out and dialysis. They found optima for the endo-PG activity at pH 3.6 and 5.4, and peaks for maceration at pH 3.0 and 5.0, measured on potato disks. At pH 4.5-5.0, the maceration seems to result from a joint action of endo-PG and PE. In addition, the authors showed that under these conditions *B. cinerea* produced both endo-PG (pH values as above), exo-PG (optimum pH 5.0), as well as PMG (with a broad optimum pH at 3.5-4.5) characterized by its attack on highly esterified pectin, and PE (optimum pH 5.0).

On a glucose-Na-polypectate-salts medium and on a pectin-salts medium, Sherwood (1964, 1966) found that *B. cinerea* produced both PG and lyase. The lyase was more active on pectin than on Na-polypectate, and more so at pH 7.2 than at 5.0. PG was more active on Na-polypectate and at pH 5.0. Damle (1952) found that *B. cinerea*, when grown in an acid environment containing an inducing substance, produced enzymes that were more

active under acid conditions than under alkaline, and that the opposite was the case when it was grown in an alkaline environment. Bateman's (1967) observations regarding *R. solani* agree with these findings.

Further, Damle (1952) showed that the macerating activity of *B. cinerea* enzymes at pH 7.6 was increasingly retarded by increasing concentrations of calcium and magnesium salts (0-0.1M), most strongly by the former. Chona (1932) not only observed retarded activity in the presence of magnesium but also in the presence of phosphate and perhaps potassium too.

The present author found the pH optimum for the lyase from *B. cinerea* to lie about 8.5, therefore he agrees with Sherwood (1966) that the activity is higher at pH 7.2 than at 5.0, and also to some extent with Tani & Nanba (1969) in that they did not find any lyase activity in the *B. cinerea* isolates when testing in the pH range from 2.7 to 6.5, although, under fair conditions, there should have been activity at pH 6.5. These two papers seem the only ones to deal with the lyase activity of *B. cinerea*, and no higher pH levels are discussed though it is known that many lyases have their pH optimum above 8.0.

On the whole it is a difficult task to make a thorough survey of all the pectolytic enzymes produced by *B. cinerea*. Tani & Nanba (1969) compiled a list of macerating and related enzyme activities in the pH range 1.5 to 6.5 and found more than twenty, which they placed in six groups according to their pH optima. However, this list is incomplete as these authors omitted all enzyme activity above pH 6.5. Further, there is a variation between the isolates both in the quality and the quantity of the enzymes, and not all the enzymes are produced by all isolates.

Tani & Nanba (1969) tested ten isolates of *B. cinerea* for macerating activity and found two enzymes able to macerate potato tuber tissue with pH optima at 2.7 and 5.5, respectively, and one enzyme that could macerate the inner bark of a Japanese plant (*Edgeworthia papyrifera* Sieb. & Zucc.) with a pH optimum at 4.5. The most active of the isolates also produced a PE and two of each of endo-PMG, endo-PG, exo-PG and cellulase. Tani & Nanba (1969) further showed that the drop in pH usually seen during the first week of growth does not apply to all the *B. cinerea* isolates on the same medium. For the most potent isolate,

the pH did not decrease to the lowest level but later reached a higher pH level than that of the other two isolates tested for this property.

The growth of *B. cinerea* on the surface of carrots is not like that of *S. sclerotiorum* under the various sets of conditions (van den Berg & Lentz 1968, van den Berg & Yang 1969, Apeland & Baugerød 1971, Djacenko 1971). *B. cinerea* never attacks carrots kept in good physiological condition (Årsvoll 1969).

6.3. Summary

This chapter outlines the knowledge of the pectolytic enzymes of *Sclerotinia sclerotiorum* and *Botrytis cinerea*, because the enzyme complexes of these fungi are those used in the majority of the author's enzyme studies (cf. Chapters 7 and 8). This outline should be compared with the results presented and with the outline of the pectolytic activity of other organisms as given in an appendix (Chapter 9).

As early as in 1886 de Bary presented a most comprehensive study of the disorganisation of plant tissues caused by *S. sclerotiorum*. He considered the disorder to be caused by a thermolabile and a thermostable factor found in front of the mostly intercellularly advancing hyphae. He proposed that the former factor was an enzyme, the latter might be oxalic acid, but he was unable to demonstrate any effect of it. Further, de Bary made the very important observation that the substances leaching out of the hyphae caused cell death as the exposed host cells were unable to plasmolyse. We are still at this stage today, though it is now definitely stated that some of the pectolytic enzymes are directly responsible for cell death (compare the appendix).

Not until more than fifty years later were studies on *S. sclerotiorum* taken up again. There has been much discussion on the production and effect of oxalic acid. It is considered that production depends on the medium and that the amount produced may be great enough to have a directly toxic effect. Other effects of oxalic acid are discussed in connection with the present author's work (cf. paragraph 8.3.3.). The pectolytic enzymes of the fungus are able to macerate plant tissues, an effect that can be greatly inhibited by Ca^{++}-ions. The enzymes are pro-

duced in different amounts by various isolates of the fungus. Most important and effective is endo-polygalacturonase (endo-PG), but probably the simultaneously produced pectinesterase (PE) acts either synergistically, if an endo-PG is the active enzyme, or as an inhibitant, if an endo-polymethylgalacturonase (endo-PMG) is the active enzyme, because the PE liberates carboxylic groups on the molecular chain. *S. sclerotiorum* isolates may also produce exo-PG, whereas it is more doubtful if they produce lyase.

The first of the very critical and most inspiring series of works on the physiology of parasitism by Brown (1915, 1936) deals with *B. cinerea* on the basis of the statement that this fungus is closely allied to *S. sclerotiorum*. Today we cannot draw conclusions from one organism to another in this way. It is clearly stated that *B. cinerea* produces oxalic acid, but it is not clear if it is produced in any quantity and under conditions comparable to those of *S. sclerotiorum*.

The very important findings of Thatcher (1939) that pathogenic fungi increase the permeability of the host tissue in advance of the hyphae involved both *S. sclerotiorum* and *B. cinera*. This author suggested that the effect was brought about by the activity of pectolytic enzymes. This has later been confirmed. Thatcher also observed that the fungi had a much higher osmotic potential in the cells than that in the cells of the hosts. Later it was found that the two fungi behave differently, as *S. sclerotiorum* may increase the osmotic potential of the cells as it increases in the host tissues, but it is not determined whether this behaviour constitutes the basis for the differences in the attacks on carrots during storage.

It was found that asparagin-containing media stimulate the production of pectolytic enzymes, both in the presence of inducer and without; consequently it is doubtful whether pectolytic enzymes, such as PE, are completely adaptive or just strongly stimulated by pectic substances.

The pectolytic enzymes and/or toxic substances produced by *B. cinerea* macerated plant tissue and killed the cells as did those of *S. sclerotiorum*. The activity was increasingly retarded with increasing amounts of calcium or magnesium salts.

Most of the experiments with *B. cinerea* were made for comparison with the pectolytic activity of other organisms, and the

differences discussed always related to polygalacturonases and pectinesterases. Only two papers discuss whether *B. cinerea* produces lyase or not. However, the information contained in these papers is not decisive because a search was only made for activity at a pH far from the optimum of lyases, which lies between 8 and 9. *B. cinerea* produces both endo-PG and exo-PG, as well as an endo-PMG and PE, so it has the ability to attack pectic substances at all levels of esterification, but not all the enzymes were produced by all isolates and under all conditions. Thus, exo-PG seems not to be produced on artificial media.

It is reported that *S. sclerotiorum* PG is equally active on pectin (9% esterified) and pectate, and concerning *B. cinerea* PG one paper reports that it has a higher activity on pectin than on pectate, while in another it is said that the enzyme behaves oppositely. This will be further discussed in connection with the present author's results.

Finally, it is noted that calcium-deficient bean plants (*Vicia faba* L.) were more susceptible to attack by *B. cinerea* and *B. fabae* than were plants receiving normal amounts of nutrients.

7. THE MACERATING EFFECT OF PECTOLYTIC ENZYMES

7.1. Introduction

On the basis of the results obtained with irradiated vegetables as discussed above, the aim of the experiments referred to in this chapter was to compare the softening effect of irradiation with the macerating effect of the enzymes from important plant pathogens, and to see if calcium could refirm the tissue or inhibit the activity of the enzymes, thereby having an effect that protects against attack.

The macerating effect of the pectolytic enzymes has almost exclusively been measured by non-mechanical methods (cf. paragraph 5.3.3.2. above) whereas the radiation-induced softening of plant tissues has been measured mainly mechanically by the methods summarized below.

Brasch & Huber (1947) mention neither the radiation dose nor the method of measuring the decrease in firmness. McArdle & Nehemias (1956) estimated the texture of carrots from tenderometer readings of 100 g of 0.25 inch diced cubes of the roots, but they did not mention the type of tenderometer they used.

Several authors report on the use of the measuring apparatus of Glegg et al. (1956) where, as mentioned above, a piston was pressed against plant tissue cylinders with a constant actuating hydraulic force.

Massey (1966, 1968) used a modified Instron Universal Testing Machine with which he was able to measure several characteristics of the plant tissues, some of which are mentioned above together with a discussion of his results. Cleland (1967) and Brown (1969) used the same kind of machine for measuring the mechanical properties of plant cells, and they gave a formula for the stress-strain instron trace:

$$D = \frac{\text{strain}}{\text{stress}} = \frac{\Delta L/L}{F/A}$$

where L is the initial length of the tissue (e.g., *Avena* coleoptile section, cabbage pith slice), and ΔL the extension, so $\Delta L/L$ gives the coefficient of extension or the strain. F is the force applied, and A the cross sectional area, so F/A gives the force per unit area or the stress. D is then the compliance or

elasticity. This formula in fact expresses Hooke's* law, which is also the principle in the tenderometer instrument itself, see below.

McClendon & Somers (1960) described an apparatus that operates on another principle for measuring the crushing loads of plant tissue slices. A weight rests on a meter stick, the distal end of which is supplied with a number of rods intended for pressing against and through the tissue. The meter stick rests on a fulcrum in balance with the weight. When the weight is moved by a synchronous motor, the rods will press against the tissue and the force used will be proportional to the readings on the meter stick.

Sawyer & Collin (1960) adapted a durometer (developed to measure hardness of rubber) to measure the hardness of potatoes. A ball tip protruded through an end plate. When pressed against the potato until touching the end plate, a dial reading (0-100) indicates how far the ball tip dents the surface of the tuber without breaking the skin. A reading of 100 means very firm. The results were given as a hardness index.

Sherwood (1966) developed a penetrometer test for the bursting strength of tissue slices and presented the results obtained as log grams.

The present author did not have access to any of the instruments mentioned above, some of which might have been useful for his experiments while others may be characterized as unfit for the purpose. However, a Volodkevich tenderometer was adapted for the purpose of measuring the maceration of carrot disks, and its mode of function is described below.

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*Robert Hooke (1635-1703) English physicist and polyhistor, who discovered that, within the elastic limits of any body, the ratio of the stress to the strain produced is constant. He also discovered plant cells.

7.2. Author's Investigations

7.2.1. Material and Methods

7.2.1.1. The Adapted Volodkevich Tenderometer. The tenderometer was developed for measuring the tenderness of foodstuffs (Volodkevich 1938), and at the Danish Meat Research Institute, for instance, it is used on meat and meat products. Thanks to the Institute, the present author was able to make a number of experiments with carrots using this instrument, but none of its pistons could be used for slices of raw carrot. Therefore, a special piston or measuring aggregate was designed (Figure 12). The lower part with the holes was placed in the holder on the tenderometer and the corresponding upper part with the rods was screwed on to the "press-bolt" of the measuring part of the instrument.

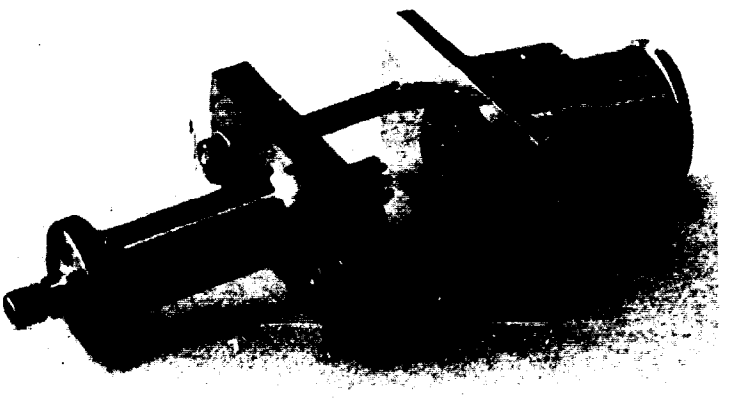

Figure 12. Piston or measuring aggregate adapted to the Volodkevich Tenderometer for measuring the crushing load of carrot tissue. Carrot disks were placed on the plate with the 9 holes, then the rods (1.5 mm diam.) with rounded tips were pressed against and through the disks by a spring-force connected with a device drawing a force-distance curve. Compare Figure 13.

The carrot disks were placed on the plate with the holes, and the rods were pressed against and through the disks. A

calibrated spring connected with the piston expands by the force of the pressure, and the expansion is transmitted to a writing device through a series of levers. The device draws a curve on a synchronously rotating paper (cf. Figure 13). This force-distance curve is proportional to the force applied, according to Hooke's law:

$$\text{Spring elongation } \Delta\ell = \frac{F \cdot \ell}{A \cdot E} \qquad (1)$$

where ℓ is the length of the spring, F the force applied, A the cross section, and E the elasticity constant.

$$\text{Force per unit area, } S = \frac{F}{A} . \qquad (2)$$

The coefficient of expansion (of the spring) is

$$\lambda = \frac{\Delta\ell}{\ell} , \qquad (3)$$

or $$\lambda = \frac{1}{E} \cdot S . \qquad (4)$$

As E and S are constants, equation (4) shows that the elongation is proportional to the stress or force per unit area (within the elastic limits of the spring, cf. Handbook of Physics, 2nd ed., McGraw Hill 1958). Examples of the force-distance curves from the experiments are given in Figure 13.

7.2.1.2. The Carrot Tissues and Microorganisms Used. Cylinders were cut out from the phloem parenchyma (p.p.) of the carrot transversal to the length using a 12 mm diam. cork-borer. The cylinder was placed in a hand microtome (Sartorius No. 24) and cut into 1 mm thick slices with a stiff, sharp razor (Ed. Wüsthof-Solingen) in order to ensure a uniform slice thickness.

Only rather large carrots could be used for these experiments, not only because of the pronounced difference in hardness between the p.p. and the core, but primarily because the cylinders had necessarily to be without traces of lateral roots as they enhance the heterogeneity of the measurements. Further, the p.p. should be thick enough for cutting 10 slices from each cylinder, or at least from the same level of the root, as the upper part of the carrot is harder than the lower.

The cylinders could be cut longitudinally, but this gives difficulties, partly because of the mentioned difference in hardness along the root, partly with avoiding the surface and

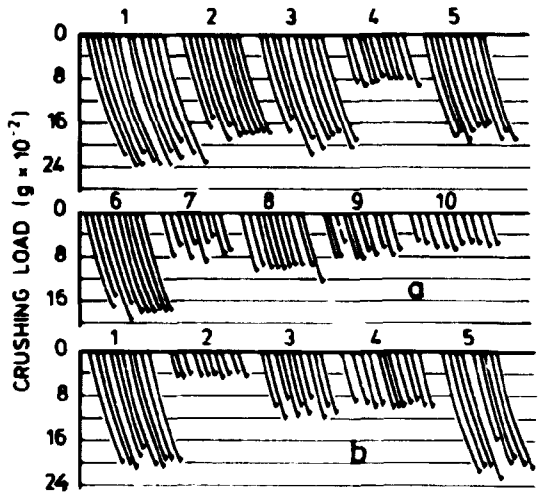

Figure 13. Examples of the force-distance curves drawn by the Volodkevich Tenderometer when measuring the crushing load of carrot tissue after immersion in the following solutions for two hours. a: 1, untreated control; 2, fungal growth medium; 3, as 2 + 0.054N $CaCl_2$; 4, *Botrytis cinerea* culture filtrate; 5, as 4 + 0.054N $CaCl_2$; 6, as 2, included for comparison; 7, 1% Fluka standardized 'pectinase'; 8, as 7 + 0.054N $CaCl_2$; 9, 0.027N oxalic acid; 10, 0.054N oxalic acid. b: 1, water; 2, *Sclerotinia sclerotiorum* culture filtrate; 3, as 2 + 0.054N $CaCl_2$; 4, as 2, but boiled; 5, as 4 + 0.054N $CaCl_2$.

core, and partly because it is a problem to get rid of the lateral root traces. Surprisingly, slices from longitudinally-cut cylinders are often harder than those from transversally-cut cylinders (Table 19). The differences between non-irradiated and irradiated carrot tissue are insignificant (Table 19).

All these errors must be considered for every carrot used. However, there are other differences that are impossible to overcome in this kind of experiment. These are the differences in firmness between the individual carrots, partly due to inheritable characters, partly due to the turgor of the tissue (cf. also Boyle et al. 1957, Kertesz 1957). However, if the carrots are used for testing all these differences in order to reduce their effect, no carrots and no time will be left for the experiment proper. The same carrot, and the same end of the carrot, is used for the different treatments at the single reaction times in order to minimize the effect of differences between carrots.

The differences in turgor are the main source of trouble, not so much for the experiment as a whole, but more with respect to what is a real or the best control. Directly, one would think that the completely untreated slices must be the right control, and so they often are because they are not significantly different from those immerged in water for some time. However, in cases where there is such a difference, it is in the author's opinion better to use the water-absorbed or the growth-medium-absorbed slices as control (cf. Table 15 in order to see the effect of water absorption). Hence, in these cases, the results will be presented relative to these treatments (= 100) (cf. Figures 14,16,22,23).

Quite often a drop in the hardness of the carrot slices occurs during the first hour of their water or growth-medium absorption. Later on in the experiment they recover their hardness. No explanation was found for this phenomenon, but it may be related to the turgor of the tissue.

For measuring the effects of treatment, the described carrot slices, 10 for each treatment and reaction time (cf. Figure 13), were immersed in 15 ml of the various solutions for the fixed time at room temperature, or for the later experiments in a Struers Cyclotherm at 25°C. During the reaction time the glasses were frequently turned over or shaken cautiously.

The 10 slices were not immersed in the solution for exactly the same lengths of time because it was necessary to drop them directly from the hand microtome into the solution, and it also took a few minutes to measure the crushing loads on the

Table 19. Crushing loads of non-irradiated and irradiated, transversally and longitudinally cut carrot disks from three experiments. Each figure is the average of 10 measurings ± SE.

No.	0 krad		12 krads	
	g crushing load when rods pressed		g crushing load when rods pressed	
	transversally	longitudinally	transversally	longitudinally
1	1668±25	1654±21	1236±43	1576±43
2	1458±28	1790±45	1540±22	1722±60
3	1682±34	2224±109	1817±35	2044±71

tenderometer. However, the procedure was always the same, for which reason this inaccuracy is random variation expressed in the standard error (SE) (Figure 13).

The carrots used for these experiments were only specially selected specimens of the varieties 'Touchon' (grown in fen soil of marine origin at the Lammefjord) and 'Fionia' (grown on very sandy soil at Sdr. Omme), or just first class carrots, washed and packed in perforated plastic bags, and bought at the greengrocers. None of the differences in the experiments, however, could be ascribed to these differences in the material used.

The carrots for these experiments were irradiated in the ^{60}Co plant at Risø. Only carrots treated with the sprout-inhibiting dose, 12 krads, were compared to non-irradiated specimens. In some of the experiments, the carrots were cut longitudinally and the one half was irradiated.

Cell-free culture filtrates from standing cultures of *Botrytis cinerea* and *Sclerotinia sclerotiorum* were used for this purpose. These fungi will produce pectin-degrading enzymes in many different liquid media. For the maceration experiments, the present author used the following two media:

Fries No. 3 (Pringle and Braun 1957)

without calcium and with reduced amounts of magnesium:

5.0 g NH_4-tartrate	30.0 g sucrose
1.0 g NH_4NO_3	1.0 mg $MnSO_4$
1.0 g KH_2PO_4	1.0 mg H_3BO_3
0.1 g $MgSO_4,7H_2O$	0.1 mg $CuSO_4$
0.1 g NaCl	0.1 mg $ZnSO_4,7H_2O$
5 mg Fe^{+++}-citrate	1000 ml water
pH 6.5	

Asparagin medium (Fernando 1937 and others)

with 20 g glucose exchanged with 10 g pectin, with reduced amounts of magnesium, and the addition of Bacto yeast extract containing 0.02% calcium:

5.0 g glucose	5.0 g KH_2PO_4
10.0 g pectin	0.2 g $MgSO_4,7H_2O$
10.0 g asparagin	2.0 g yeast extract
pH 6.5	1000 ml water

The latter medium was used with several modifications for most of the experiments in this work. It was originally used because it was recommended for the production of pectolytic exo-cellular enzymes from the fungi in question (Fernando 1937, Fernando & Stevenson 1952, Mishra 1953, Ashour 1954), and later used because it proved very excellent for the purpose. Though the medium was prepared with a pH of 6.5 and sterilized by heat, no interfering degradation products from the pectin were detectable (compare Albersheim 1959).

Calcium was avoided and magnesium was reduced in order to make it possible to study the effect of calcium under different conditions. The fungi grow very well and produce plenty of enzymes without calcium and with small amounts of magnesium. By removing the last trace of magnesium, the fungi practically stop growing (Skou 1971a VIII).

For all experiments with pectolytic enzymes, the fungi were grown as standing cultures on the surface of 40 ml of liquid medium (largest free surface) in 150 ml Erlenmeyer flasks in the dark at 23-25°C after inoculation with a hardly visible amount of aerial mycelium. The production of pectolytic enzymes sets in, increases with the growth, and reaches its maximum after 8-12 days. Cultures of this age were often used for the experiments, but experiments were also made with young and older cultures.

In the experiments studying the effects of calcium on the storability of carrots, the calcium salt was used according to weight. Therefore, in order to make all the experiments comparable, the same concentration, or multiples of it, was used in all later experiments giving the special figures seen for normality. These concentrations were also used for other chemicals so as to give the same ionic strength.

The pH was adjusted as desired but generally without the addition of any buffer, because most of the buffers usable in such experiments will interact with the calcium making both more or less inactive. Further, the intention was to make the experiments as close to natural conditions as possible to determine what the effect of calcium would be if used in practice.

As it was found important also to study the maceration of carrot slices when containing a very reduced amount of calcium, the slices were treated with 0.1M EDTA (compare Letham 1958,

1960, and Ginzburg 1961, who used 0.07, 0.003-0.34 and 0.1M concentrations, respectively) in order to remove the calcium from the tissue. The EDTA reactions were carried out at pH 8, which should give a good chelating effect with Ca^{++} according to Letham (1960), but also pH 5 was used because this was important for the later treatment with enzymes. These treatments should not disturb the cells themselves (Letham 1958, 1960, Ginzburg 1961). Room temperature was used, and for further treatment the carrot slices were rinsed in water and transferred to the other solutions in question.

The culture filtrates of the fungi were compared with Fluka standardized pectinase purum batch No. 76290.

7.2.2. Results and Discussion

First, the question arose of which buffer to use when the intention was to relate the experiments as closely as possible to natural conditions. Further, the buffer should not remove Ca^{++}-ions from the reaction mixture or tissue, neither should it affect the tissue in other ways or the enzymatic activities.

The next question was whether Ca^{++} should be applied as a pretreatment to imitate the use of $CaCl_2$ prior to storing of the carrots, or whether it should be present during the process of reaction. Further, what would be the differences in maceration if the tissue was made deficient in or enriched with Ca^{++}?

A series of experiments was performed to solve these problems. The results (Table 20) show an apparent effect (not always detectable, see below) of irradiation both on the buffered carrot slices and on those treated with the culture filtrate. McIlvaine's standard buffer solution apparently had an effect on the maceration both with and without the presence of culture filtrate, and even in 1/4 ionic strength. For these reasons, and because this and most other buffers usable in these experiments will react with the Ca^{++}-ions and thereby disturb the effect of both buffer and calcium, I decided to omit buffers. This gave, of course, some decrease in pH during the two-hour reaction time, but not so much as to enhance maceration, because the pH of the carrot slices themselves is between 6 and 7 against the initial pH 5 in the reaction mixture, which is without reacting materials other than the carrot slices.

Table 20. Texture of carrot disks measured as grammes crushing load.

Treatment for 2 hours	0 krad				12 krads			
	Further treatment for 1 hour in				Further treatment for 1 hour in			
	McIlvaine's buffer solution, pH 5		S. sclerotiorum culture filtrate, pH 5		McIlvaine's buffer solution, pH 5		S. sclerotiorum culture filtrate, pH 5	
	g	relative	g	relative	g	relative	g	relative
Control	(1682±34	100)*			(1817±35	100)		
0.1M EDTA, pH 8	916±11	54	636±27	38	620±16	34	376±16	21
0.1M EDTA, pH 5	670±16	40	636±16	38	318±12	18	224± 5	12
0.0272N $CaCl_2$	1790±76	106	1836±49	109	1734±48	95	1576±54	87
McIlvaine's standard buffer solution 1/4 strength, pH 8	1538±51	91	1330±33	79	1272±37	70	1312±16	72
McIlvaine's standard buffer solution 1/4 strength, pH 5	1474±73	88	1134±28	67	1372±56	76	1024±34	56

*( ) means no further treatment.

In contrast to what is stated in the literature (Letham 1958, 1960, Ginzburg 1961), EDTA had a greater effect at pH 5 than at pH 8. As expected from the literature (cf. Kertesz 1951, Skou 1971a VIII), the $CaCl_2$-treatment increased the firmness of the non-irradiated carrot slices, but it did not have this effect on the irradiated slices. Further, the $CaCl_2$ inhibited the macerating activity of the culture filtrate from *S. sclerotiorum*.

Figure 14 shows the results of an experiment where pretreatment with EDTA, in order to remove calcium from the carrot tissue, is followed by a treatment with $CaCl_2$ to see how fast the

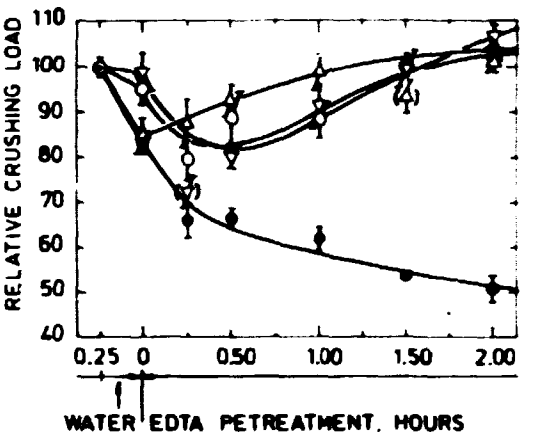

Figure 14. Effect of treatment of carrot tissue with 0.027N $CaCl_2$ after softening by pretreatment with EDTA for increasing periods of time. ◉ : untreated control; ● : EDTA pretreatment only; ○, ▽, and △ : in $CaCl_2$ for 0.25, 0.50 and 1.00 hours, respectively. The SE is given.

tissue regains the firmness lost through the EDTA treatment and if it reaches the normal level. The results given are relative to the untreated control. When pretreated with EDTA only, the curve shows the expected drop in firmness. The 3 curves for calcium treatment for 0.25, 0.50 and 1 hour show - by the distance to the EDTA curve - that the longer the pretreatment with EDTA (more than 0.5 hour), the faster is the increase in firmness, i.e. a more rapid uptake of calcium. All three calcium

post-treatment curves reach the same level, which is somewhat higher than that of the control and the same as seen when normal carrot tissues are treated with $CaCl_2$. The pretreatment with EDTA for 15 and 30 min obviously only made relatively small changes in the tissue, hence the following calcium treatment for 15 and 30 min also had a correspondingly small effect. However, it is surprising that the firmness of the 1 mm thick carrot slices can reach the peak level in so short a post-treatment with calcium after 2 hours of EDTA-treatment. The curve for 1 hour of $CaCl_2$ post-treatment has a tendency to increase faster at the beginning than the curves for 15 and 30 min, but this is insignificant.

These results agree with the literature in that EDTA-treated tissues at pH 8 behave as normal tissues, but what does such an increase in firmness and the extra calcium taken up mean for the effect of pectolytic enzymes from the carrot pathogens? And what will be the effect on the tissues from which the calcium is removed by EDTA? An experiment performed to elucidate these questions gave the results presented in Figure 15. As may happen in this kind of experiment, the carrots lost firmness when kept in water for 2 hours, because of the turgescence and of their prehistory. The loss in 0.1M EDTA for 2 hours was naturally much more pronounced (Figure 15 signature 1-3).

After these pretreatments, series of slices were transferred to a culture filtrate of *B. cinerea* where they proceeded to lose in firmness (Figure 15 signatures 4 and 7). Those pretreated with EDTA lost firmness much faster than those pretreated with water. The explanation for this could either be an after-effect of EDTA in the tissue, or that EDTA facilitated the maceration by removing Ca^{++} from the tissue. In order to solve this problem, slices were placed in 0.027N solutions of $CaCl_2$ or NaCl for half an hour after the pretreatment with water (Figure 15 signatures 5 and 6) or EDTA (Figure 15 signatures 8 and 9). For the water-treated carrots, a little increase appeared in firmness both in the $CaCl_2$ and in the NaCl solutions, and when the slices were transferred to the *B. cinerea* culture filtrate the two curves were equal and parallel to the original curve without the salt treatment. This means that a pretreatment with Ca^{++} of water-absorbed carrot slices had no effect on the subsequent macerating activity of the *B. cinerea* culture filtrate. In the EDTA-treated

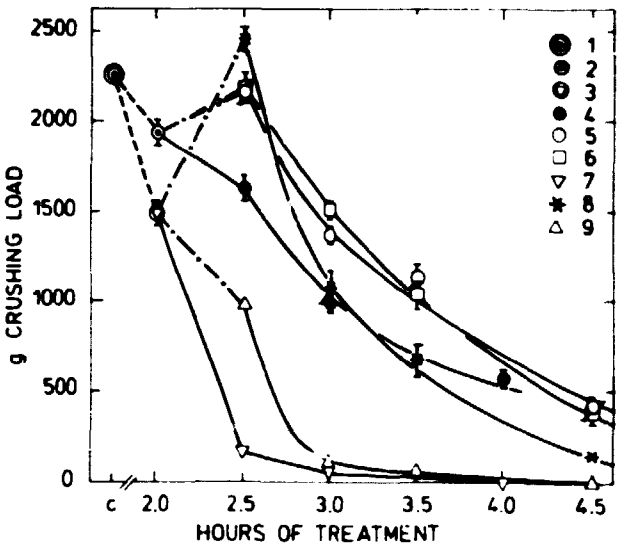

Figure 15. Effect on the activity of *Botrytis cinerea* culture filtrate of pretreatment of carrot tissue with 0.027N NaCl, with 0.027N $CaCl_2$ in order to enhance the calcium content of the tissue with calcium, and with 0.1M EDTA in order to exhaust the tissue for calcium. The treatments follow the scheme set up below where the letters refer to the symbols on the curves.

Hours of treatment								
0	1: control							
2	2: water			3: EDTA				
0.5	4: culture filtrate	5: $CaCl_2$	6: NaCl	7: culture filtrate	8: $CaCl_2$	9: NaCl		
2	culture filtrate	culture filtrate	culture filtrate	culture filtrate	culture filtrate	culture filtrate		

Note the effect of the addition of NaCl and $CaCl_2$ after the pretreatment with water or EDTA. Note also the parallel displacement of the curves after the EDTA pretreatment, and that the curves after 0.5 hours in NaCl and $CaCl_2$ following the water pretreatment are identical. The SE is given, but in some cases it is smaller than the symbols.

carrots, a further drop in firmness appeared during half an hour (dotted and dashed part of the curve, Figure 15 signature 9) in the NaCl-solution, although it was less pronounced than for the slices in the culture filtrate. This drop is, at least partly, supposed to be an after-effect of the EDTA-treatment. When transferred to the cultu. filtrate, the curve for these slices ran parallel to that of the original EDTA-pretreated slices. Slices transferred from the EDTA solution to the $CaCl_2$-treatment for half an hour (dotted and dashed part of the curve, Figure 15 signature 8) increased very strongly in firmness to a level above the original, as expected. The curve for the following treatment with the *B. cinerea* culture filtrate, however, ran almost parallel to the two other curves from the EDTA pretreatment. This means that neither in this case did a Ca^{++} pretreatment alter the macerating activity of the *B. cinerea* culture filtrate. The greater firmness or calcium content in the tissue after the EDTA-$CaCl_2$-treatments did not alter the maceration rate as was expected; but although the tissue behaves normally in a purely physical way, this does not imply that it is also unchanged with respect to its biochemistry.

Based on these results the following experiments were performed without pretreatments of any kind. The experiments were set up comparing non-irradiated carrots with carrots treated with 12 krads under the influence of strong and weak solutions of macerating enzymes from *S. sclerotiorum*, *B. cinerea* and Fluka standardized pectinase, as indicated above, and of $CaCl_2$ in order to see how it affects the activity of the enzymes.

Figures 16 and 17 show the results with culture filtrates from *S. sclerotiorum*. There was a pronounced difference in the activity of the culture filtrates in the two experiments, but no detectable effect of irradiation. Without culture filtrate, the $CaCl_2$ had no or a weak firming effect on the carrot slices. The presence of $CaCl_2$ in the culture filtrate clearly reduced the maceration, but much less in the more active culture filtrate than in the weaker.

Figures 18 and 19 present the same situation with the *B. cinerea* culture filtrate, although Figure 18 only refers to non-irradiated carrots. The results are in complete agreement with those in which the *S. sclerotiorum* culture filtrate was used (cf. Figures 16 and 17). Doubling the $CaCl_2$ concentration gave

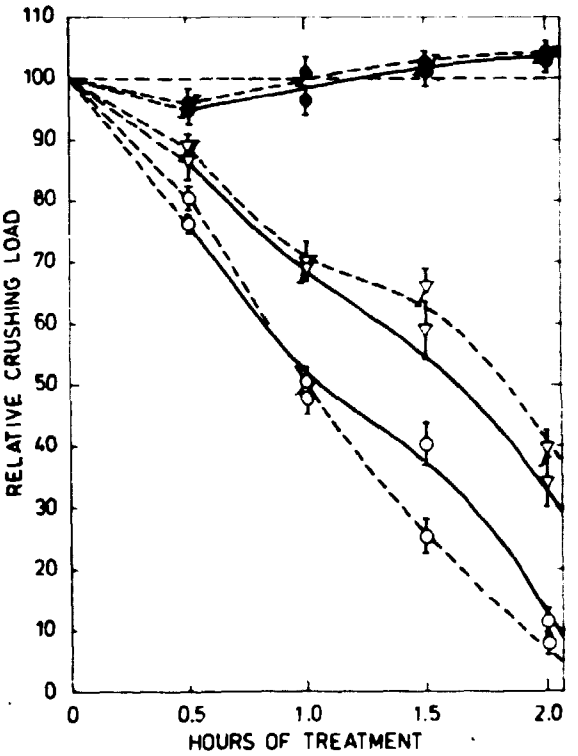

Figure 16. The effect of calcium on the macerating activity of *Sclerotinia sclerotiorum* culture filtrate on irradiated and non-irradiated carrot tissue. The curves are drawn relative to that of the sterile growth medium.——: non-irradiated;– –: irradiated; ● : growth medium + 0.027N $CaCl_2$; ○ : culture filtrate; ▽ : culture filtrate + 0.027N $CaCl_2$. The SE is given.

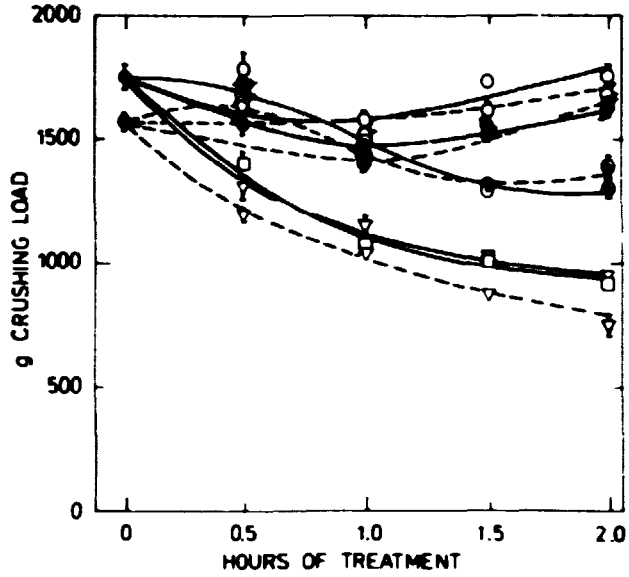

Figure 17. The effect of calcium on the macerating activity of a weak *Sclerotinia sclerotiorum* culture filtrate on irradiated and non-irradiated carrot tissue.——: non-irradiated;— —: irradiated; ● : growth medium; ○ : growth medium + 0.027N $CaCl_2$; ▽ : culture filtrate; ◉ : culture filtrate + 0.027N $CaCl_2$; □ : culture filtrate + 0.027N NaCl. Note the parallel displacement of the curves for irradiated and non-irradiated carrots that results from different hardness only. Note also the absence of any effect of the addition of NaCl. The SE is given.

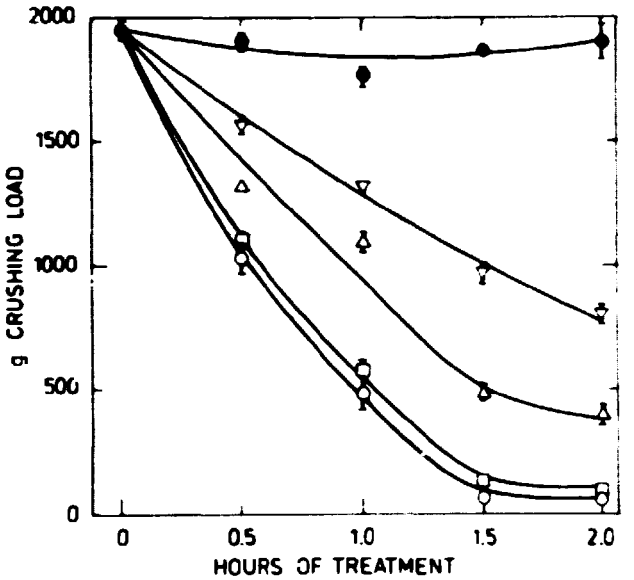

Figure 18. The effect of NaCl and $CaCl_2$ concentration on the macerating activity of a very active *Botrytis cinerea* culture filtrate on carrot tissue. ● : growth medium; ○ : culture filtrate; △ : culture filtrate + 0.027N $CaCl_2$; ▽ : culture filtrate + 0.054N $CaCl_2$; □ : culture filtrate + 0.027N NaCl. The SE is given.

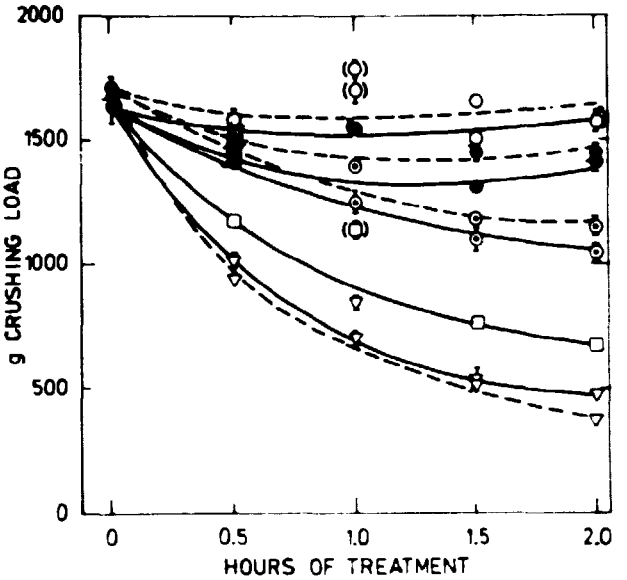

Figure 19. The effect of NaCl and $CaCl_2$ on the macerating activity of a weakly active *Botrytis cinerea* culture filtrate on irradiated and non-irradiated carrot tissue.——: non-irradiated;– –: irradiated; ● : growth medium; ○ : growth medium + 0.027N $CaCl_2$; ▽ : culture filtrate; ◉ : culture filtrate + 0.027N $CaCl_2$; □ : culture filtrate + 0.027N NaCl. Note the parallel displacement of the curves for irradiated and non-irradiated carrots that results from different hardness only. The SE is given.

a greater inhibition of maceration, but it did not stop the activity of the very active culture filtrate.

The difference between the weaker and the more active culture filtrates regarding the inhibitory effect of $CaCl_2$ could possibly be ascribed to differences in the amount of oxalic acid produced in the growth medium. This problem will be described below.

There was no detectable effect of irradiation (Figure 19). The results after a one-hour treatment were too high (or with too little effect) because the carrot material used was heterogeneous.

Figures 20 and 21 show the results with 0.5% and 1% Fluka standardized pectinase, respectively. If the differences in the initial firmness of the carrot slices is considered, it is surprising that there is so little difference in the macerating effect of the two concentrations of 'pectinase'. $CaCl_2$ had no effect at all on the activity of Fluka pectinase.

In several cases (see Figures 17-21) the effect of culture filtrate or 'pectinase' with 0.027N NaCl was included in the experiments for comparison with the $CaCl_2$ treatment. Generally, the curve for the NaCl treatment is so close to those for the culture filtrates or 'pectinase' that it may be concluded that the effect of $CaCl_2$ is not just a salt effect.

There may be other reasons for the differences in the effect of $CaCl_2$ in connection with its use in the culture filtrates or the 'pectinase'. In the culture filtrates, the effect of $CaCl_2$ increases with concentration but decreases with increasing activity of the filtrate, while it has no effect at all in the 'pectinase' solutions. The reason could be different proportions of the macerating enzymes in the culture filtrates, but it could also be a question of different amounts of oxalic acid produced by the fungi during growth, and then with the action of this alone or synergistically with the enzymes. The Fluka standardized pectinase is produced by other organisms and does not contain oxalic acid. These facts may be the reason for the absence of a calcium effect.

Figure 22 presents results confirming the above statements about the effect of calcium on culture filtrates and on Fluka pectinase. In this case, 0.054N $CaCl_2$ completely stopped the activity of the *B. cinerea* culture filtrate, but it had no effect on the activity of the 'pectinase'. The same experiment

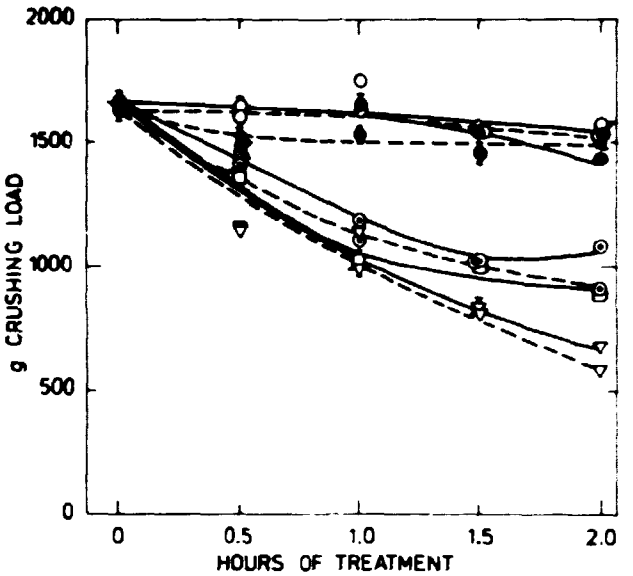

Figure 20. The effect of NaCl and $CaCl_2$ on the macerating activity of 0.5% Fluka 'pectinase' on irradiated and non-irradiated carrot tissue.——: non-irradiated;— —: irradiated; ● : growth medium; ○ : growth medium + 0.027N $CaCl_2$; ▽ : 'pectinase'; ◉ : 'pectinase' + 0.027N $CaCl_2$; □ : 'pectinase' + 0.027N NaCl. The SE is given.

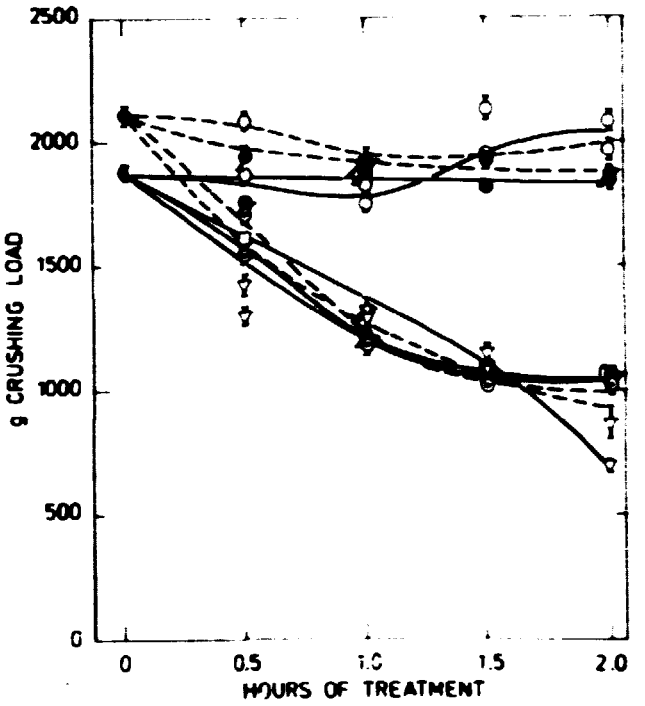

Figure 21. The effect of NaCl and $CaCl_2$ on the macerating activity of 1.0% Fluka 'pectinase' on irradiated and non-irradiated carrot tissue.——: non-irradiated;– –: irradiated; ● : growth medium; ○ : growth medium + 0.027N $CaCl_2$; ▽ : 'pectinase'; ◉ : 'pectinase' + 0.027N $CaCl_2$; □ : 'pectinase' + 0.027N NaCl. Note the parallel displacement of the curves for irradiated and non-irradiated carrots that results from different hardness only. The SE is given.

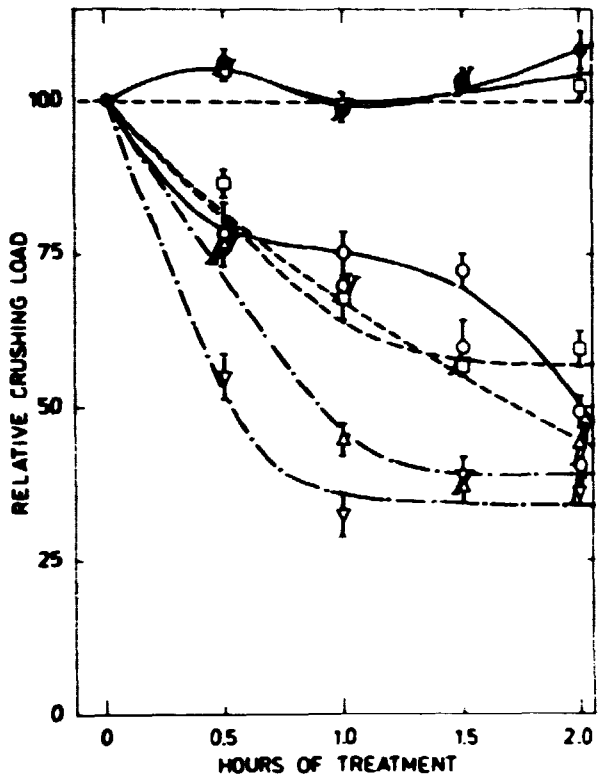

Figure 22. The effect of calcium on the macerating activity of *Botrytis cinerea* culture filtrate and of 1% Fluka 'pectinase' compared with the effect of oxalic acid on carrot tissue. The curves are drawn relative to that of the sterile growth medium. —●—: growth medium + 0.054N $CaCl_2$;—○—: culture filtrate;—□— : culture filtrate + 0.054N $CaCl_2$; – –○– –: 'pectinase';– –□– –: 'pectinase' + 0.054N $CaCl_2$;—·—△—·— : 0.027N oxalic acid;—·—▽—·— : 0.054N oxalic acid. The SE is given.

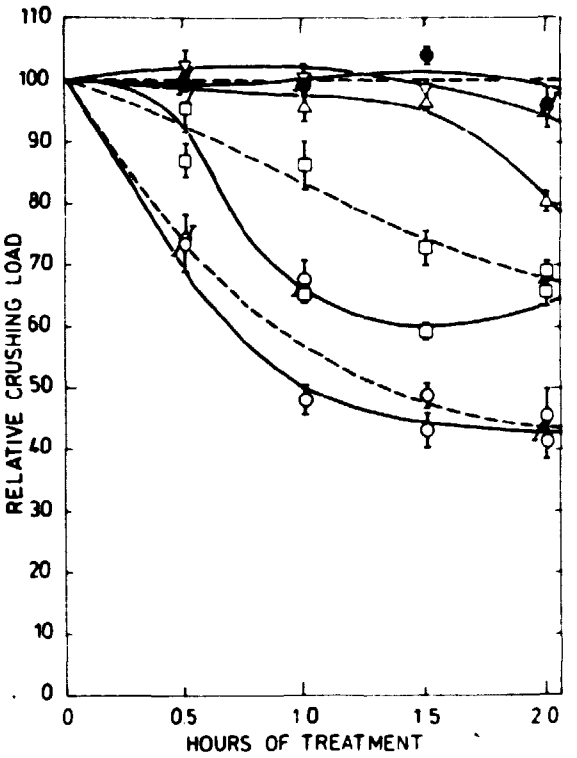

Figure 23. The effect of calcium and of boiling on the macerating activity of *Botrytis cinerea* culture filtrate on irradiated and non-irradiated carrot tissue. The curves are drawn relative to that of the sterile growth medium.——: non-irradiated;– –: irradiated; ● : growth medium + 0.027N $CaCl_2$; ○ : culture filtrate; ▢ : culture filtrate + 0.027N $CaCl_2$; △ : boiled culture filtrate; ▽ : boiled culture filtrate + 0.027N $CaCl_2$. The SE is given.

showed a very strong macerating or softening effect of oxalic acid. This effect increases with concentration and is characteristic in that it ceases within an hour's treatment.

Figure 23 shows that boiling this *B. cinerea* culture filtrate removed the greater part of its activity, and that the addition of 0.027N $CaCl_2$ to the boiled culture filtrate removed the rest of the macerating activity. The effect of 0.027N $CaCl_2$

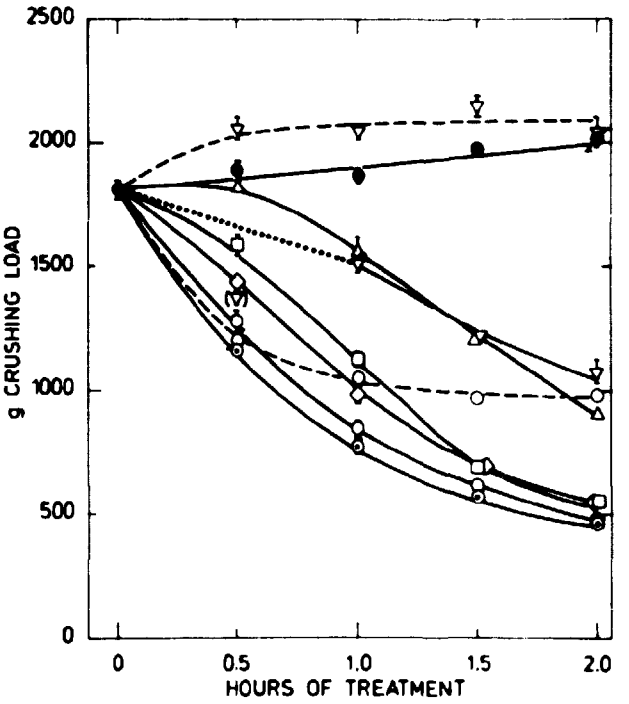

Figure 24. The effect of NaCl, of increasing concentrations of $CaCl_2$, and of boiling on the macerating activity of *Sclerotinia sclerotiorum* culture filtrate on carrot tissue.——: fresh culture filtrate; — — : boiled culture filtrate; ●: water; ○: culture filtrate; ◉: culture filtrate + 0.014N $CaCl_2$; □: culture filtrate + 0.027N $CaCl_2$; △: culture filtrate + 0.041N $CaCl_2$; ▽: culture filtrate + 0.054N $CaCl_2$; ◇: culture filtrate + 0.054N NaCl. The SE is given.

on the activity of the raw culture filtrate was larger than that which removed the rest of the activity from the boiled filtrate. This means that calcium affected both the thermolabile and the thermostable fraction, or said in another way, the two fractions act additively or synergistically and are both affected by calcium. The effect of irradiation is questionable.

In this experiment the effect of the thermostable factor, which is most likely oxalic acid, is not very pronounced. From other experiments, reported later on, it is known that *B. cine-*

tea is a late and weak producer of oxalic acid. This is in agreement with the results of the experiment under present discussion.

In contrast to *B. cinerea*, *S. sclerotiorum* is a fast and strong producer of oxalic acid (see the series of experiments discussed in paragraph 7.3.3.). Figure 24 presents results with increasing concentrations of $CaCl_2$ in the culture filtrate, and a comparison of the raw and the boiled culture filtrate. The lowest calcium concentration had no effect and the two highest concentrations had almost the same effect. Boiling reduced the macerating activity somewhat and gave a curve of the same shape as those from the oxalic acid treatment (compare Figures 22 and 23). The addition of 0.054N $CaCl_2$ to the boiled culture filtrate completely stopped the macerating activity and instead was able to increase the firmness of the carrot slices. In this case the calcium treatment left a white precipitate of calcium oxalate in the culture filtrate.

A salt concentration of 0.054N NaCl in the culture filtrate had little effect.

7.2.3. Summary

Various instruments for measuring maceration were discussed, and the adaptation of the Volodkevich tenderometer for use on carrot slices was described.

In the present author's experiments the intention was to relate the experiments as closely as possible to natural conditions. This led to methods without the use of buffers because they influence plant tissues as well as pectin, calcium and the enzymes.

EDTA treatment softens carrot tissue because it removes Ca^{++}-ions and other cations from the cross linkages of the pectic substances in the middle lamellae. $CaCl_2$ treatment has the opposite effect. Carrot slices softened by EDTA treatment rapidly regain their firmness when treated with $CaCl_2$, and they reach a similar level of firmness irrespective of the degree of softness obtained by the EDTA treatment; i.e., the softer the tissue, the higher the rate of regain of firmness. Pretreatment with $CaCl_2$ did not alter the macerating effect of the *B. cinerea* culture filtrate, neither did a pretreatment with EDTA followed by a $CaCl_2$ treatment alter the macerating rate. Thus Ca^{++}-ions

must be present in the solution during the reaction in order to achieve the effect.

$CaCl_2$ reduced the maceration but much less in the more active culture filtrates than in the weaker ones. Doubling the $CaCl_2$ concentration further inhibited the maceration but without stopping it. $CaCl_2$ had no effect at all on the macerating activity of Fluka pectinase.

A closer examination of the macerating effect of the culture filtrates showed that at the least they consist of a thermolabile and a thermostable component, because boiled filtrates still have a macerating effect although they lose much of their activity. The larger the activity of the unboiled filtrate, the larger also the rest effect after boiling and vice versa. The macerating effect of the thermostable component could easily be stopped by $CaCl_2$ in the reaction mixture. Without $CaCl_2$, the reaction curve proceeds in a characteristic manner as the macerating rate changes drastically after an hour, whereas the unboiled filtrate proceeds to macerate the tissue. This indicates that the thermostable component could be oxalic acid, because experiments with this acid give the same shape of curve and the differences in effect may result from the production of different amounts of oxalic acid. Later the thermostable component was identified as oxalic acid (cf. paragraph 8.3.3.).

The two components act additively or synergistically and they are both strongly inhibited or prevented in the presence of Ca^{++}-ions. It was later shown that *B. cinerea* is a weak and late producer of oxalic acid in contrast to *S. sclerotiorum*. Fluka pectinase is produced by other organisms and does not contain oxalic acid.

Culture filtrates of *S. sclerotiorum* and *B. cinerea* macerated carrot tissues without any detectable difference between non-irradiated and irradiated material.

Only now and then, and almost randomly, was it possible to detect an effect on the crushing load after irradiation with 12 krads, and the many treatments in this series of experiments disclosed no differences between the non-irradiated carrot slices and those exposed to 12 krads that could be ascribed to an effect of the irradiation.

8. AUTHORS'S INVESTIGATIONS ON THE PRODUCTION AND EFFECT OF PECTOLYTIC ENZYMES AND OXALIC ACID AS INFLUENCED BY CALCIUM

8.1. Introduction

Many microorganisms produce from one to several pectolytic enzymes and oxalic acid, and there is detailed information in the literature about the effect of the isolated, individual enzymes as influenced by various sets of environmental conditions including the effect of calcium. This effect on more or less purified enzymes (polygalacturonase and pectin lyase) has been studied by many authors, as referred to in paragraph 5.5 and in the Appendix. Much less is known about the effect that results from the simultaneous influence of enzymes and oxalic acid in nature when a pathogen attacks a certain host, or when the enzymes are left to act on pectic substances after growth on artificial media.

It is therefore the intention of this chapter to elucidate the effect of calcium on the activity of pectolytic enzymes and oxalic acid during their simultaneous action and interaction as it may take place when the pathogens degrade the pectic substances either in vitro, or in situ in the middle lamellae of vegetable tissue. It is on this total effect that an external supply of calcium could reveal its possible practical use.

Under these conditions, the normal enzymological procedure cannot be applied, as it is impossible to use purified enzymes for determination of parameters such as specific activity, initial rate of activity (possibly apart from some experiments with pectinesterase), maximal velocity at substrate saturation ($V_{max.}$), and Michaelis constant (K_m), equal to the substrate concentration when the velocity is half $V_{max.}$ (cf. Anonymous 1965a).

8.2. Material and Methods

Most of the experiments were performed with enzymes from *Botrytis cinerea* and *Sclerotinia sclerotiorum* that cause severe soft-rot in carrots. Supplementary experiments were performed with a few other carrot pathogens causing other types of rot during the storage of carrots (cf. Årsvoll 1969, Jørgensen &

Jensen 1975). The fungal isolates used are listed in Table 21.

The culture filtrates used for measuring the enzyme activity in vitro were prepared as described in Chapter 7. Also the carrots used for experiments on the enzyme activity of healthy and diseased (sterilized and inoculated with the pathogens) carrots were of the types described in this chapter.

Enzyme activity was measured on commercial pectic substances that were characterized by a series of analyses based mainly on conventional methods, e.g. those of Rahman & Joslyn (1953) and Henglein (1955) for determination of the various components of the pectic molecular chain. The results are given in Table 22.

The free carboxyl groups were determined by dissolving the pectic substances in boiled, distilled water and titrating with 0.1N NaOH using phenolphthaline as indicator.

For determination of the esterified carboxyl groups, the pectic substances were saponified in 1.0N NaOH followed by back-titration with HCl, which gives the total amount of carboxyl groups, and by subtraction of the free carboxyl it gives the amount of esterified carboxyl groups. From these data it should be possible to calculate the galacturonic acid units and the amount of other components by the formula of Henglein (1955).

The water content was calculated after drying the pectic substances at 65°C for 24 hours, and the content of ashes after calcination at 550°C. The traces of carbonate ions may be an artifact.

The total N was determined by Kjeldahl analyses, and the ammonia and 'protein' content was calculated on the basis of the amount of basic amino acids (lysin, histidin and arginin) and NH_3 found in the amino acid analyzer.

The content of cations was determined by atomic absorption spectrophotometry (Jarrell-Ash), and the anions detected by simple conventional methods (cf. page 60).

Genu pectin NF (see Table 22) was used for most of the experiments both for growth of the microorganisms and for determination of pectolytic activity. It differs clearly from the other pectins by its low content of divalent cations and its relatively high content of sodium ions. Further it has the highest content of ashes and the lowest water content.

On this basis the calcium content of the reaction mixture without addition of salts was calculated to be 0.248 mg/l. This

Table 21. Fungi used in the experiments with pectolytic enzymes.

Botanical names	Common names	Isolate and habitate
Botrytis cinerea Pers. ex Fr.	grey mould	No. 43 isolated from carrot, 60 from raspberry, 70, 73, 74 from strawberry, 80 from cauliflower, 720 from wheat, 931 from air contamination
Chalaropsis thielavioides Peyronel	black lesions of carrots	No. 1023 isolated from carrot
Mycocentrospora acerina (Hartig) Deighton (syn. *Centrospora acerina* (Hartig) Newhall)	black crown, side rot, licorice rot	No. 1022 isolated from carrot
Sclerotinia sclerotiorum (Lib.) de By.	Sclerotinia soft rot, watery soft rot, white rot	No. 246, 793, 794, 975 isolated from carrot

Table 22. Characterization of the commercial pectic substances used in the experiments.

	% water	% ashes in dry matter	N-compounds, %		% of various components in the pure pectic substances			Contents stated in commercial catalogues and from personal communication**)
			'protein'	NH_3	free acid	methyl ester	galacturonic acid*)	
Genu citrus pectin type N.F. 52751	4.53	2.89	1.17	0.29	13.01	71.70	79.89	ashes < 7%, water max. 10%, galacturonic acid min. 78%
Fluka pectin from *Citrus decumana* (purum) batch 76280	8.10	1.60	2.94	0.38	12.74	84.28	91.34	ashes ≤ 2%
Fluka pectin from apples (purum) batch 76282	7.85	1.27	1.12	0.28	23.61	65.66	84.85	ashes ≤ 2%
BDH pectin from apples, 250 grade No. 38052	9.48	1.42	1.70	0.28	15.52	68.60	79.50	sulphated ashes 1%, CH_3O - max. 16.3%
Fluka pectic acid (purum) batch 76300	9.69	2.51	0.49	0.01	81.42	16.85	97.13	ashes 2-4%

	NH_3 meq/g · 10^2	Na^+ meq/g · 10^2	K^+ meq/g · 10^2	Ca^{++} meq/g · 10^2	Mg^{++} meq/g · 10^2	monoval. cations, total meq/g · 10^2	divalent cations, total meq/g · 10^2	Cl^-	SO_4^{--}	PO_3^{--}
Genu citrus pectin N.F.	17.1	42.1	4.5	0.55	0.41	64.1	1.0	-	+	traces
Fluka pectin from *Citrus decumana*	22.4	9.9	3.2	10.92	1.65	35.5	12.6	-	+	"
Fluka pectin from apples	16.5	3.8	7.4	7.08	2.47	27.7	9.6	-	+	"
BDH pectin from apples	16.5	7.2	3.0	12.82	2.39	26.7	15.2	-	+	"
Fluka pectic acid	0.59	36.4	1.1	1.15	0	38.1	1.2	-	+	"

*) Calculated from the formula of Henglein (1955)

**) Fluka 1975 p. 372; BDH 1975, p. 209; The Copenhagen Pectin Factory, Ltd.

corresponds with 0.9% of the calcium in a 0.027N $CaCl_2$ reaction mixture.

Pectinesterase (PE). Ten ml culture filtrate was added to 0.5% Genu pectin N.F. to give 50 ml reaction mixture. This was titrated with 0.1N NaOH at room temperature (i.e. 22°C) in a continuous titrator (Radiometer type TTT 1a) keeping the pH at 4.75 during the reaction.

Reducing end-group methods and the thiobarbituric acid (TBA) method were used for measuring the polygalacturonase (PG) activity.

The reducing end-groups produced during growth on pectin as carbon source and when the culture filtrates act on pectin solutions were qualitatively detected by means of the Fehling reaction method, whereas quantitative analyses were made with a modification of the Willstätter-Schudel iodometric titration method (Veibel 1954, Kertesz 1955, Patel & Phaff 1959) with iodide and thiosulphate using starch as indicator by the following procedure: 25 g 0.5% pectin - or pectic acid - solution plus 10 ml culture filtrate or enzyme solution were rapidly adjusted to pH 6.0 on the titrator and made up to 50 ml with distilled water. The reaction mixture was then immediately placed in a Cyclotherm at 30°C. At exact time intervals 5 ml samples were transferred to 150 ml standard (normal) ground Erlenmeyer flasks for analyses. 5.0 ml 0.1N iodide solution was admixed, after which 8.0 ml 0.1M Na_2CO_3 was added drop by drop under continuous stirring. After the last drop of Na_2CO_3 and the addition of 2 ml 4N H_2SO_4 and 50 ml distilled water, the flasks remained closed with glass stoppers for exactly 30 minutes. Finally, the reaction mixture was titrated with a 0.05N $Na_2S_2O_3$ solution until pale yellowish in colour, and further until colourless after the addition of some drops of a starch solution.

The method was verified with galacturonic acid.

The thiobarbituric acid (TBA) method was used for measuring the polygalacturonase (PG) and the lyase activity. The basis of this method is the formation of coloured reaction products that have absorption maxima at 515 and 547 nm for PG and lyase, respectively (Neukom 1960, Starr & Moran 1961, 1962). The TBA-method exists in many modifications of which that used by Sherwood (1966) is perhaps closest to the method used by the present author.

To 25 g of pectin - or pectic acid - solution (normally, to achieve a final concentration of 0.5%) was added 5 ml distilled water and 10 ml culture filtrate or enzyme solution while stirring with a magnetic rod, which process continued while the pH was adjusted. For the reasons discussed above, buffers were omitted. Instead, the pH of the reaction mixture was frequently adjusted at the titrator. This does not disturb the process, because the pH optima for the enzymes are too wide-ranged to change as a result of a small variation in pH. After adjustment of the pH, the reaction mixture was placed on the Cyclotherm at 30°C for 1 hour or more. At the end of the exact reaction time, samples were taken for analysis. The reaction may be stopped by boiling, by $ZnSO_4$ precipitation (Ayers, Papavizas & Diem 1966), or by the addition of N HCl; the last method was found to be very efficient and easiest to handle. To 5.0 ml of the reaction mixture was then added 1.5 ml N HCl, 0.5 ml distilled water, and 3 ml 0.04M 2-thiobarbituric acid under vigorous agitation. The samples were then placed in a 95°C hot water bath for 1 hour, cooled in running water and made up to a final volume of 10 ml. Controls were handled in the same way. The solution was centrifuged at approximately 10,000 g for 10 minutes and the colour intensity of the clear supernatant was measured in a Unicam double beam spectrophotometer (SP 800) over the wave range 480-560 nm.

The method was verified with galacturonic acid (Figure 25), which showed that the increase in absorbance is proportional to the concentration of galacturonic acid. The reaction, however, is not fully explained and it did not come to an end when stopped, but the heterogeneity increased if the heating continued too long. We found the best reaction time to be 1 hour at the 95°C used.

The amounts of galacturonic acid units liberated by the polygalacturonase activity are calculated from the absorbance at 515 nm according to the linear regression curve of Figure 25, whereas the amount of $\Delta_{4,5}$-deoxygalacturonic acid liberated by the lyase activity is given as the absorbance at 547 nm because no standard curve could be made. In cases where the absorbances of the two substances interact, they are separated according to the formula of Bartlet & Smith (1960),

$$Y = \frac{N}{\sigma\sqrt{2\pi}} \cdot e^{-\frac{1}{2}(x/\sigma)^2} ,$$

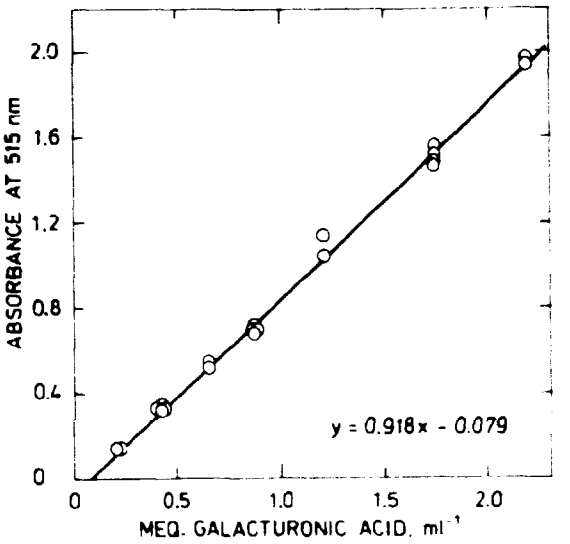

Figure 25. Standard curve for the galacturonic acid-2-thiobarbituric acid reaction at 95°C for 1 hour.

which gives the height of every point on a Gaussian distribution curve. The absorption curves for the two substances were calculated to fit this distribution (compare Figure 36).

The height Y for peak a, at distance x_a *from the middle* value, is found by substituting $N/\sigma\sqrt{2\pi}$ by h'_a, so that, for a curve with two overlapping peaks, we find the formula,

$$Y = h_a e^{-\frac{1}{2}(x_a/\sigma_a)^2} + h_b e^{-\frac{1}{2}(x_b/\sigma_b)^2},$$

from which the single peaks may be calculated as

$$h_a = \frac{h'_a - h'_b e^{-\frac{1}{2}(x_b/\sigma_b)^2}}{1 - e^{-\frac{1}{2}[(x_a/\sigma_a)^2 + (x_b/\sigma_b)^2]}}$$

and the same can be done for h_b, and not necessarily by approximation as proposed by Bartlet & Smith (1960).

In a few cases, where some "shadows" occurred with the curves, the base line for the absorption was calculated according to the operation instructions for the spectrophotometer

(Anonymous 1966).

The oxalic acid produced during the growth of the fungi was measured in the following way (cf. e.g. Pucker, Wakeman & Vickery 1941, Wolf 1955, Bateman & Beer 1965):

1 ml culture filtrate is added to 4 ml $CaCl_2$-acetate buffer prepared as follows:

A: 25 g $CaCl_2$ (water-free, calculated)
250 ml glacial acetic acid
250 ml distilled water
B: 330 g Na-acetate
500 ml distilled water
A and B are mixed.

The reaction mixture is left overnight in centrifuge glasses. Then it is centrifuged at 3,000 g for 10 minutes, the supernatant discarded, and the precipitate suspended in 5 ml 5% acetic acid saturated with Ca-oxalate. After further centrifugation, and discarding the supernatant again, the precipitate is dissolved in 5 ml 4N H_2SO_4 and quantitatively transferred to a beaker by washing with a further 5 ml 4N H_2SO_4. The solution is heated to 90-100°C in a water bath and titrated with 0.01N $KMnO_4$ to a very weak, pink colour that lasts for several minutes.

Other organic acids with calcium salts of low solubility, e.g. fumaric, malic, and succinic acid, may be produced during growth (cf. Vega, Corsini & le Tourneau 1970). However, tests made by the present author showed that 1 ml 1% of these acids gave no detectable precipitate by this method, whereas 1 ml < 10^{-3}% oxalic acid could be detected. Although these acids may bind calcium in the same way as does oxalic acid, they are of little significance in connection with the present work unless produced in rather large amounts.

8.3. Results and Discussion

8.3.1. Pectinesterase Activity

PE hydrolyzes the methoxyl groups of pectic substances (cf. Figure 7). It has been said that the enzyme is produced adaptively (cf. Chapter 6). However, it is easy to demonstrate its production on media without pectin, but production increases strongly in the presence of pectin (Figures 26-28). The aspara-

gine medium, as described above, but often used with pectin as the only other source of carbon, was found to be excellent for enzyme production - a fact also found by others, as mentioned earlier.

Great differences have been found between strains of *B. cinerea* regarding the production of pectolytic enzymes including PE (Figure 26), but it is not known whether these differences arise from keeping the strains in culture or if they occur in nature. Nevertheless, strain No. 43, used for most of the experiments, has maintained its high enzyme production for several years.

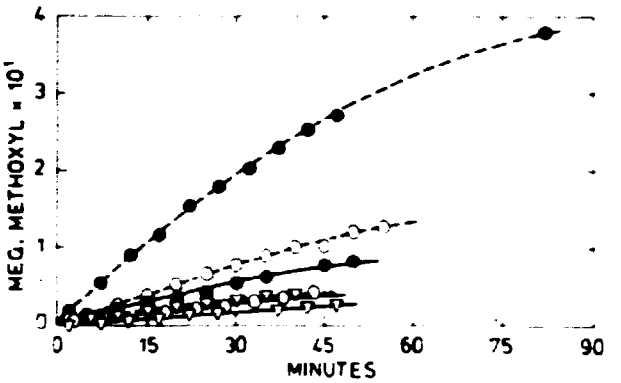

Figure 26. Activity of pectinesterase from three isolates of *Botrytis cinerea* after growth on Czapek-Dox medium (——) or asparagine medium with 0.25% pectin (- - -). Measured as the equivalents of methoxyl groups liberated from a pectin solution. ● : No. 43 isolated from carrot; ○ : No. 60 isolated from raspberry; ▽ : No. 720 isolated from wheat root.

At the same time the curves indicate that calcium stimulates the PE activity initially, but the reaction ceases on a lower level of hydrolysis (Figures 27 and 28). This was further analyzed.

Not only do the Ca^{++}-ions stimulate PE activity, but also the Na^{+}-ions have a stimulatory effect, although there is a characteristic difference in the effect of the two cations. Even though NaCl clearly increases the velocity of the reaction, the maximal level of hydrolysis is practically the same as with-

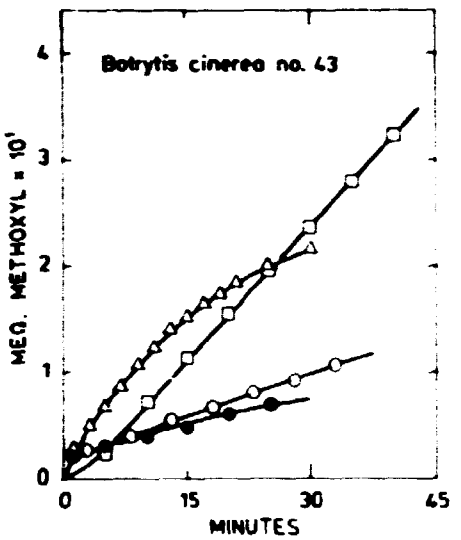

Figure 27. The effect of pectin in the growth medium on the production of pectinesterase from *Botrytis cinerea* (No. 43), and the effect of calcium on the equivalents of methoxyl groups liberated from a pectin solution.
Without pectin in the growth medium: ● : no salt added; ○ : 0.054N $CaCl_2$ added. With pectin in the growth medium: □ : no salt added; △ : 0.054N $CaCl_2$ added.

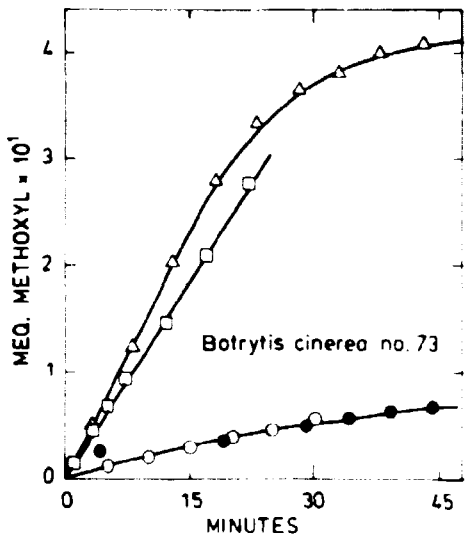

Figure 28. The activity of *Botrytis cinerea* isolate No. 73 from strawberry under the same conditions as described for figure 27 above.

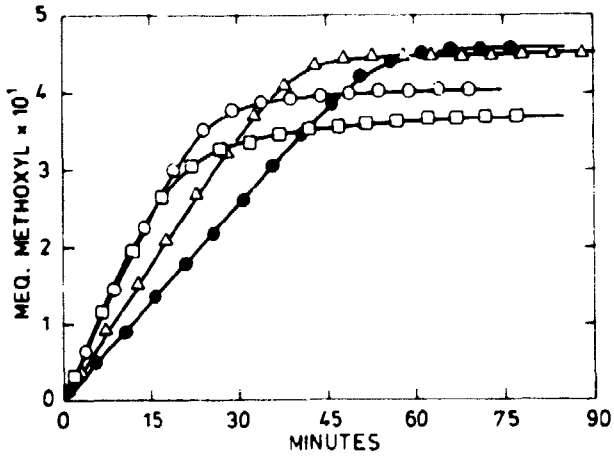

Figure 29. The effect of NaCl and $CaCl_2$ on the activity of pectinesterase from *Botrytis cinerea* (No. 43) as measured by the equivalents of methoxyl groups liberated from a pectin solution. ● : without salt added; ○ : 0.027N $CaCl_2$; □ : 0.054N $CaCl_2$; △ : 0.027N NaCl.

out added salts (Figure 29, Table 23). For the same ion concentration with $CaCl_2$, the velocity is clearly higher than for NaCl, but the maximal level of hydrolysis is considerably lower and it is further lowered with increasing concentration of $CaCl_2$ (Figure 29, Table 23). Using Fluka pectic acid, which is not completely exhausted of methoxyl groups (cf. Table 22), the picture is exactly the same, but on a much lower level (Figure 30, Table 23).

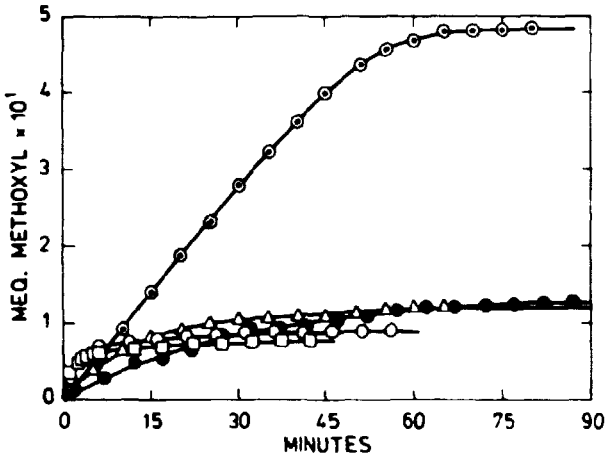

Figure 30. The effect of NaCl and $CaCl_2$ on the activity of pectinesterase from *Botrytis cinerea* (No. 43) as measured by the equivalents of methoxyl groups liberated from a solution of Fluka pectic acid (compare Table 22) and, for comparison, the activity of the same culture filtrate on a pectin solution (Genu N.F.) is inserted. ● : without salt added; ○ : 0.027N $CaCl_2$; □ : 0.054N $CaCl_2$; △ : 0.027N NaCl; ◉ : on pectin solution without salts added.

The PE from *S. sclerotiorum* behaves similarly whether produced in culture or in carrot tissue. Healthy carrot tissue exhibits only weak PE activity (Figure 31, Table 23).

The reason for the shape of the curves may be discussed. In the first part of the curves, the amount of free carboxyl groups produced is proportional with the reaction time and it is therefore regarded as a zero-order reaction which means that

Table 21. Pectinesterase activity. Initial velocity and the maximum amount of acid split with enzymes from different sources and under different conditions.

Refers to Figure No.	Organism and strain	Origin of enzyme	Addition salts	Initial velocity (ml 0.1 N alkali per ml enzyme per hour)	Maximum amount of acid split (ml 0.1 N alkali per ml enzyme)	Remarks
32	Sclerotinia libertiana No. 63	asparagine medium + pectin	-salts	[illegible]	4.4	[illegible]
[illegible]	" " [illegible]	Czapek-Dox medium*	-salts	[illegible]	[illegible]	
[illegible]	" " [illegible]	asparagine medium	-salts	[illegible]	[illegible]	
[illegible]		Czapek-Dox medium	-salts	[illegible]	[illegible]	
26	" " No. 67	asparagine medium	-salts	[illegible]	[illegible]	
[illegible]		Czapek-Dox medium	-salts	[illegible]	[illegible]	
26	" " No. 120	asparagine medium	-salts	[illegible]	[illegible]	
27	Sclerotinia libertiana No. 63	asparagine medium + pectin	-salts	[illegible]	[illegible]	
			+0.354% $CaCl_2$	[illegible]	[illegible]	
27	" " No. 63	asparagine medium	-salts	[illegible]	[illegible]	
			+0.354% $CaCl_2$	[illegible]	[illegible]	
28	" " No. 73	asparagine medium + pectin	-salts	[illegible]	[illegible]	Values for a certain time for calculation of max. hydrolysis
			+0.354% $CaCl_2$	[illegible]	4.2	
28	" " No. 73	asparagine medium	-salts	[illegible]	[illegible]	
			+0.354% $CaCl_2$	[illegible]	[illegible]	
29	" " No. 63	asparagine medium + pectin	-salts	[illegible]	4.4	in pectin
			+0.027% $CaCl_2$	[illegible]	4.14	" "
			+0.354% $CaCl_2$	[illegible]	[illegible]	" "
			+0.027% NaCl	[illegible]	[illegible]	" "
30	" " No. 63	asparagine medium + pectin	-salts	[illegible]	[illegible]	in pectin
			-salts	[illegible]	[illegible]	in pectic acid
			+0.027% $CaCl_2$	[illegible]	[illegible]	" " "
			+0.354% $CaCl_2$	[illegible]	[illegible]	" " "
			+0.027% NaCl	[illegible]	[illegible]	" "
31	Sclerotinia libertiana No. 70	asparagine medium + pectin	-salts	[illegible]	4.64	in pectin
			+0.354% $CaCl_2$	[illegible]	[illegible]	" "
		juice from attacked carrot tissue	-salts	[illegible]	[illegible]	" "
			+0.354% $CaCl_2$	[illegible]	[illegible]	" "
		juice from healthy carrot tissue	-salts	[illegible]	[illegible]	" "
35	Fluka 'pectinase'	commercial, 1%	-salts	[illegible]		max. hydrolysis cannot be calculated
			+0.027% $CaCl_2$	[illegible]		
			+0.054% $CaCl_2$	[illegible]		
			+0.027% NaCl	[illegible]		
35	Sigma 'pectinase'	commercial, 1.25%	-salts	[illegible]		max. hydrolysis cannot be calculated
			+0.027% $CaCl_2$	[illegible]		
			+0.054% $CaCl_2$	[illegible]		
			+0.054% NaCl	[illegible]		

*) Czapek-Dox liquid medium; a sucrose-inorganic-salts medium, cf. DIFCO Manual p. 285, 1942.

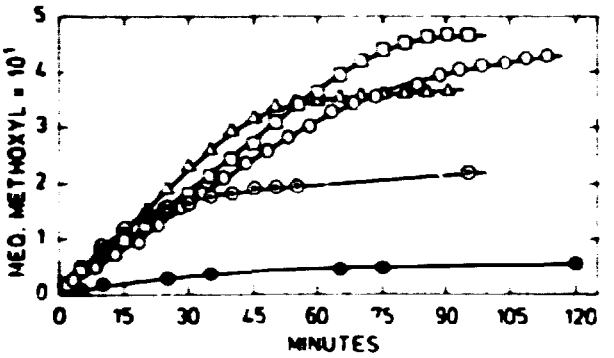

Figure 31. The effect of $CaCl_2$ on the activity of pectinesterase from *Sclerotinia sclerotiorum* (No. 794) in juice from attacked carrots and in culture filtrate as measured by the equivalents of methoxyl groups liberated from a pectin solution. ● : juice from healthy carrots; ○: juice from attacked carrots; ◉ : juice from attacked carrots + 0.054N $CaCl_2$; □ : culture filtrate; △: culture filtrate + 0.054N $CaCl_2$.

the reaction is unaffected by the concentration of the reacting substances (cf. Daniels & Alberty 1955; Table 23 and Figures 26-32). After some time, depending on the conditions, the reaction rate slows down and ceases at a maximal level of hydrolysis, the causes of which are supposed to be complex and involving inaccessibility to the remaining substrate rather than due to exhaustion (cf. Jansen & MacDonnell 1945, Solms & Deuel 1955, Wood 1967, Morris 1971). The cessation of the reaction has nothing to do with the concentration of the substrate, as only approximately one third of the methoxyl groups are hydrolyzed even at the highest maximal level of hydrolysis without the addition of salts (Figures 29-31); neither does it have anything to do with the destruction of the enzyme because the addition of more enzyme after 80 minutes did not result in any increased hydrolysis (Figure 32). Regardless of whether $CaCl_2$ is added or not, and whether the methoxyl content is high as in pectin, or very low as in pectic acid (see Table 22), the shape of the curves is always the same. Only the initial rate and the maximal level of hydrolysis differs. The results may be regarded as an expression of the simultaneous action of the pectolytic enzymes

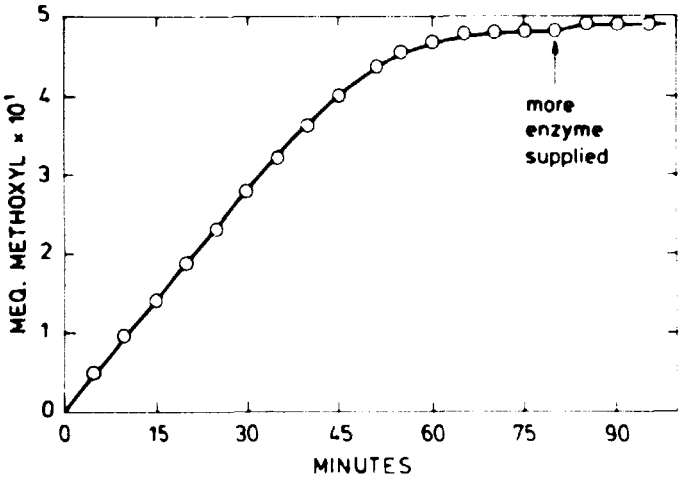

Figure 32. The activity of pectinesterase from *Botrytis cinerea* (No. 43), and the effect of a further supply of enzyme (culture filtrate) after the activity of the first supply has ceased following hydrolysis of about one third of the methoxyl groups in the pectin solution.

present. On the other hand, the activity of exo-pectate lyase - free of other enzymes - from *Clostridium multifermentans* exhibited exactly the same curve shapes under the influence of calcium (MacMillan & Vaughn 1964). The interaction may be stimulatory or inhibitory. De-esterification enhances the PG-activity (cf. e.g. Jansen & MacDonnell 1945), but inhibits the PMG activity, and the simultaneous action of PG and PE may enhance the de-esterification compared to the activity of PE alone (cf. e.g. Jansen, MacDonnell & Jang 1945). The latter finding is supposed to result from a rapid breakdown of the pectic acid produced that is inhibitory to PE. Also the activity of the PG has been found inhibitory to PE (Solms 1954). Solms & Deuel (1955) stated that hydrolysis by PE was always incomplete, leaving about 10% of the methoxyl groups, and Wood (1960) noted that even at optimal conditions some ten per cent of the pectin will always remain esterified. The explanation for this may lie in the results of Solms & Deuel (1955), who found that trigalacturonic acid trimethylester, digalacturonic acid mono- and dimethylester,

and monogalacturonic acid methylester were not attacked by plant PE. Further, they found the rate of hydrolysis dependent on the distribution of the methoxyl groups along the molecular chain, and it was indicated in this connection that a methoxyl group having a free carboxyl group adjacent on one or both sides is easier hydrolysed than when adjacent to methoxyl groups.

The addition of $CaCl_2$ (Figures 29-31) is so different from the addition of NaCl that it cannot just be a salt effect. Perhaps the stimulatory effect of Ca^{++}-ions on the PE activity is based on the masking of the carboxyl groups and thus of their inhibitory effect on hydrolysis, as described by Solms & Deuel (1955). However, the Ca^{++}-ions binds two carboxyl groups. This may have an inhibitory effect on the further hydrolysis of the methoxyl groups of the smaller pieces of the pectin molecule, in analogy with the findings of Solms & Deuel (1955) with the tri- and di-galacturonic acid methylesters. At the same time, these compounds remain from the hydrolysis of pectin by endo-PG, and all the different PG's (exo-PG, endo-PG, PMG) were inhibited in the presence of Ca^{++}-ions, but the pectin lyase showed no activity at pH 4.75. Simultaneous action of the three enzymes or enzyme groups at different pH levels is seen in Table 24 (compare Figures 36 and 37). PG and PE exhibited a very

Table 24. Activity of pectolytic enzymes from *Botrytis cinerea* for 3 hours at 30°C at different pH levels.

pH	Polygalacturonase activity, galacturonic acid liberated		Pectin lyase activity, $\Delta_{4:5}$-deoxygalacturonic acid liberated	Pectinesterase activity, methoxyl groups hydrolyzed
	absorbance at 515 nm, corrected	meq per ml	absorbance at 547 nm, corrected	maximal hydrolysis, meq x 10^1
4.0	1.71	1.95	0	5.00
5.0	1.54	1.76	0.49	7.00
6.0	1.14	1.33	0.96	6.50
7.0	0.57	0.70	1.33	3.75
8.0	0.48	0.61	1.56	1.85
9.0	0.46	0.59	1.48	3.58

high activity at the low pH values. At pH 6 all three enzymes showed considerable activity and at higher pH levels there were only traces of PG activity, an increasing pectin lyase activity, and a decreasing activity of PE until pH 8.0. The secondary increase in the hydrolysis of the methoxyl groups at pH 9.0 is at least partly due to basic hydrolysis (saponification).

It is not completely clear at which pH level saponification starts to become considerable; it may be at about pH 7.

The level of this hydrolysis at pH 8-9 is always higher with culture filtrates from *B. cinerea* than with those from *S. sclerotiorum*. There is no explanation of this, but *B. cinerea* may produce a second methoxyl hydrolyzing agent active at the high pH level, or saponification may be facilitated by the activity of the pectin lyase, as parallel results were found when studying the influence of calcium. Such parallelism was not found when using a culture filtrate from *S. sclerotiorum* that does not exhibit pectin lyase activity (Table 25). However, the parallelism is not always consistent, but as the calcium has little effect on the pectin lyase activity and obviously has an effect on the secondary hydrolysis or saponification of the methoxyl groups, the inconsistency may be due to fluctuations in the NaOH titre (Figures 33 and 34). Another possibility is that *B. cinerea* produces other acid degradation products of pectin at the high pH level.

Finally, the activity of PE from Fluka and Sigma 'pectinase' differs clearly from that of *B. cinerea* and *S. sclerotiorum* (Figure 35), but also their PG's behave differently, for which reason the results need not only depend on the PE activity. The Fluka standardized pectinase purum (batch No. 76290) is of plant origin (personal communication 1976), whereas that from Sigma originates from *Aspergillus niger* (Sigma catalogue 1974); compare the enzyme activity of *A. niger* referred in the Appendix.

Table 25. Activity of pectolytic enzymes at increasing $CaCl_2$ concentrations at pH 8.5. Control at pH 5.0 and without $CaCl_2$.

$CaCl_2$	pH	Polygalacturonase activity Absorbance at 515 nm	Pectin lyase activity Absorbance at 547 nm	Pectinesterase activity Maximal hydrolysis meq × 10^1
Botrytis cinerea No. 43:				
0	5.0	1.22	zero	4.70
0	8.5	traces	0.39	1.25
0.027N	"	"	0.43	1.50
0.054N	"	"	0.44	1.65
0.108N	"	"	0.45	1.75
0.216N	"	"	0.41	1.95
Botrytis cinerea No. 931:				
0	5.0	1.76	traces	7.10
0	8.5	shoulder	1.62	2.23
0.027N	"	"	1.71	2.70
0.054N	"	"	1.74	2.68
0.108N	"	"	1.53	2.48
0.216N	"	"	1.35	2.23
Sclerotinia sclerotiorum No. 794:				
0	5.0	1.43	zero	5.73
0	8.5	zero	zero	0.55
0.027N	"	"	"	0.45
0.054N	"	"	"	0.63
0.108N	"	"	"	0.73
0.216N	"	"	"	0.63

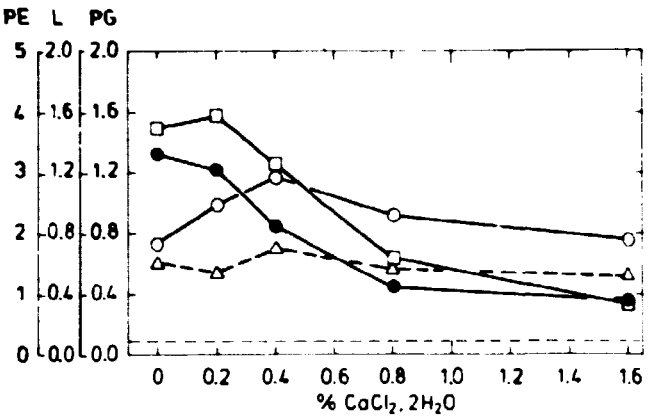

Figure 33. The effect of $CaCl_2$ concentration on the activity of the pectolytic enzymes from *Botrytis cinerea* at pH 5.0, i.e., close to the optimum for pectinesterase (PE) and polygalacturonase (PG), and at pH 8.5, i.e., close to the optimum for pectin lyase (L). The PE is measured as the equivalents of methoxyl groups liberated from the pectin solution, L is measured as the absorbance of the reaction products at 547 nm, and PG is measured as the absorbance of the reaction products at 515 nm and given as meq galacturonic units liberated (compare Figure 24). ● : PG activity at pH 5.0; ○ : L activity at pH 8.5; □ : PE activity at pH 5.0; △ : methoxyl groups hydrolyzed at pH 8.5.

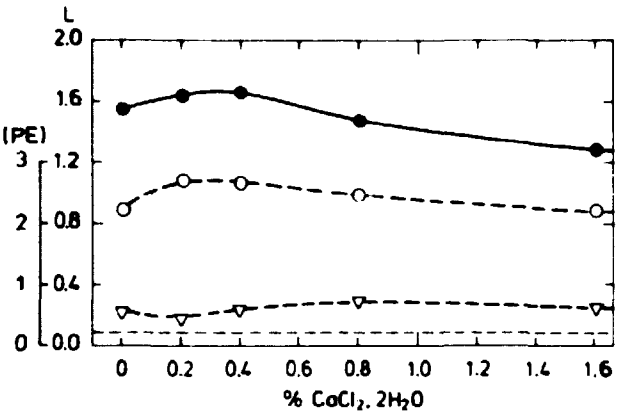

Figure 34. The effect of $CaCl_2$ concentration on the activity of pectin lyase (L) from a *Botrytis cinerea* (No. 931) culture filtrate at pH 8.5 compared with the simultaneous hydrolysis of methoxyl groups from a pectin solution, and with a culture filtrate from *Sclerotinia sclerotiorum* (No. 794), which does not produce pectin lyase. —●—: pectin lyase from *B. cinerea*;——○——: methoxyl group equivalents hydrolyzed from the *B. cinerea* reaction mixture;——▽——: methoxyl group equivalents hydrolyzed from the *S. sclerotinia* reaction mixture.

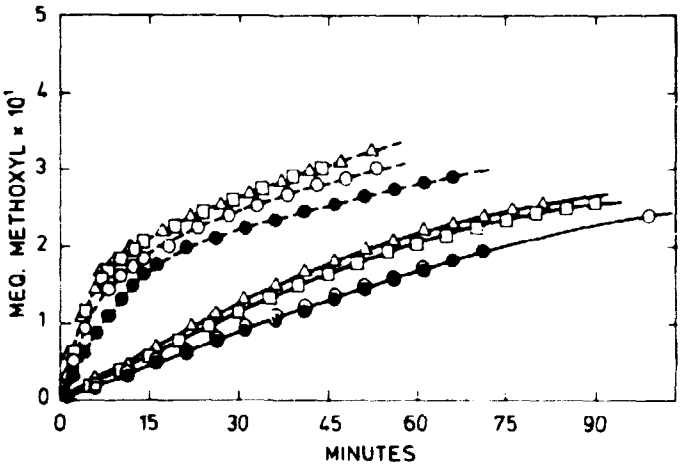

Figure 35. The effect of NaCl and $CaCl_2$ on the activity of pectinesterase from commercial 'pectinase' as measured by the equivalents of methoxyl groups liberated from a pectin solution. ——: 1% Fluka 'pectinase';– – –: 0.25% Sigma 'pectinase'; ● : without salt added; □ and △: 0.027N and 0.054N $CaCl_2$, respectively; ○: 0.027N and 0.054N NaCl for Fluka and Sigma 'pectinase', respectively.

8.3.2. Activity of Polygalacturonases and Lyases

The mode of activity of these enzymes is given in paragraph 5.2 and Figure 7.

It is easy to confirm the fact stated by several authors about different fungi that pectin and pectic acid used as a carbon source stimulate the production of the pectolytic enzymes and that glucose is a very poor carbon source in this context, while the production of pectolytic enzymes on various combinations of these compounds is difficult to explain. This may be connected with the use of asparagine in the basic medium, as this amino acid in itself induces the production of pectolytic enzymes (cf. e.g. Fernando 1937, Fernando & Stevenson 1952, Mishra 1953, Brown & Wood 1953, Ashour 1954). Table 26 gives an example of the differences in enzyme activity with pectin, pectic acid or glucose as the other carbon source. From this it

Table 26. Amount of reaction products from the activity of pectolytic enzymes from *Sclerotinia sclerotiorum* and *Botrytis cinerea* grown on different media with and without calcium ions added.

	Growth medium	Reaction products accumulated*) in the growth medium		Measured enzyme activity of					
				polygalacturonase		pectin lyase		pectinesterase	
		galacturonic acid, meq per ml	$\Delta_{4,5}$-deoxygalacturonic acid, absorbance at 547 nm	galacturonic acid, meq per ml		$\Delta_{4,5}$-deoxygalacturonic acid, absorbance at 547 nm		meq methoxyl groups hydrolyzed per 50 ml reaction mixture	
				pH 5.0	pH 8.5	pH 5.0	pH 8.5	pH 5.0	pH 8.5
S. sclerotiorum	pectin**)	0.36	0	1.10	0.14	0	0	5.05	0.50
	"	0.40	0	0.65		0		6.47	
	" + Ca^{++}***)	0.39	0.21	1.82	0.11	0	0	6.80	0.55
	" + Ca^{++}	0.12	0.04	1.77		0		6.90	
	pectic acid	0.62	0	0.69		0		2.90	
	" " + Ca^{++}	0.11	0.03	0.77		0		4.30	
	glucose	0	0	0.15		0		4.90	
	" + Ca^{++}***)	0.13	0.04	0		0		0.30	
B. cinerea	pectin**)	0.13	0.04	1.87	0.50	trace	1.23	6.95	1.50
	"	0.35	0.65	0.53		0		5.30	
	" + Ca^{++}***)	0.29	0.54	2.65	0.57	0	1.28	7.35	2.25
	" + Ca^{++}	0.30	0.75	0.90		0		6.30	
	pectic acid	0.33	0	0.47		0		2.30	
	" " + Ca^{++}	0.17	0.08	0.46		0		1.95	
	glucose	0.11	0.02	0.30		0		3.00	
	" + Ca^{++}***)	0.10	0.01	0.19		0.26		0.15	

*) see Table 28; **) another experiment in which only pectin and pectin + Ca^{++} were compared;
***) very poor growth.

is also obvious that the presence of Ca^{++} in the growth medium enhances the production or activity of polygalacturonase (PG) and of pectinesterase (PE), but hardly of the pectin lyase. Neither is there an obvious influence on the accumulation or breakdown of reaction products in the growth medium.

Both *S. cinerea* and *S. sclerotiorum* exhibit fairly good growth on galacturonic acid as sole source of carbon, but as this acid accumulates in the growth medium its assimilation is slower than the liberation from the pectic substances. It has not been tried to grow the fungi on $\Delta_{4:5}$-deoxygalacturonic acid, but, as this compound accumulates and disappears in the growth medium in the same way as the liberated galacturonic acid, it is considered to follow the same pattern (Table 26). The accumulation and disappearance of these reaction products is temperature-dependent, as is the production and activity of the pectolytic enzymes, which further seem quite stable in the culture media, though more at 15°C than at the higher temperatures (Table 27).

As soon as there is a measurable growth, the reaction products start to accumulate. At this time it is not yet possible to detect the enzyme activity by the methods used.

The growth media are adjusted to pH 6.5 without the use of a buffer as explained under "Material and Methods". In the first few days of growth the pH drops below 4 for both fungi - a slightly larger drop for *S. sclerotiorum* than for *S. cinerea*. Soon after, the pH in the *S. cinerea* cultures starts to rise again, and about two weeks after inoculation the pH is between 7 and 8. *S. sclerotiorum* behaves almost similarly, but it takes several days before the secondary rise of the pH starts. This phenomenon is well known in the literature, and in the present investigations it is regarded as connected with the lowering of the C/N ratio during growth and a breakdown of the asparagine. No great influence on the production and stability of the pectolytic enzymes has been observed as a result of these fluctuations and levels of pH.

A very different effect of pH is that on the activity of the pectolytic enzymes. Polygalacturonase had greatest activity at or below pH 5, pectinesterase had an optimum at about pH 5, and the lyase at about pH 8-8.5 (Table 24). For the hydrolysis of the methoxyl groups, a secondary increase occurs at the high

Table 27. Activity of pectolytic enzymes from *Sclerotinia sclerotiorum* and *Botrytis cinerea* at different temperatures and ages measured by the accumulation of reaction products in the growth medium or produced on pectin.

		Reaction products accumulated in the growth medium*						Measured enzyme activity of								
		galacturonic acid, meq per ml			$\Delta_{4,5}$-deoxy-galacturonic acid, absorbance at 547 nm			polygalacturonase, galacturonic acid, meq per ml				pectin lyase, $\Delta_{4,5}$-deoxy-galacturonic acid, absorbance at 547 nm	pectinesterase, methoxyl groups hydrolyzed meq per 50 ml reaction mixture			
								pH 5.0			pH 8.5	pH 8.5	pH 5.0			pH 8.5
Growth temperature, °C \ Age of cultures in days		12	19	36	12	19	36	12	19	36	19	36	12	19	36	36
S. sclerotiorum	15	0.39	0.15	0.13	0	0.11	0.04	0.35	0.62	1.72	0.15	0.11	0.85	2.15	6.15	1.20
	25	0.12	0.15	0.12	0.02	0.06	0.03	0.59	1.48	1.46	0.15	0.10	5.55	5.65	5.75	0.40
	28	0.38	0.23	0.13	0	0	0.03	0.65	1.52	1.11	0.15	0.11	2.00	6.60	6.60	0.70
B. cinerea	15	0.37	0.47	0.13	0.72	0.94	0.04	1.07	0.61	1.22	0.42	0.96	4.95	3.70	5.15	1.55
	25	0.48	0.13	0.13	0.84	0.04	0.04	0.96	1.51	1.12	0.55	1.35	3.45	5.85	5.00	1.45
	28	0.52	0.13	0.13	0.72	0.04	0.04	1.02	1.85	0.87	0.60	1.50	4.90	6.85	3.75	2.05

*) Per ml reaction mixture in order to be able to compare the figures to those of the measured activity. For calculation of the amounts accumulated in the growth medium, the figures should be multiplied by the dilution factor 5. An meq below 0.15 and an absorbance below 0.08 are doubtful.

pH value as discussed above. An example of the activity of the enzymes from *B. cinerea* is given in Figure 36 and from a series of measurements in Figure 37. All three enzymes exhibit a most considerable activity about pH 6, which is also the normal pH value of many vegetable products including carrots. If the pH decreases below this level, the activity of PG will increase, and if the pH increases above this level, the lyase activity will increase.

The specificity of polygalacturonase and lyase on various fractions of pectic substances is discussed in the literature (cf. the survey of the literature on *S. sclerotiorum* and *B. cinerea* above and the Appendix). Some of the enzymes are more active on pectin than on pectic acid, others behave in the opposite manner, and still others seem to be equally active on both substances. For *S. sclerotiorum* and *B. cinerea* the present author has found no literature giving comparisons of the enzyme activity on pectins and/or pectic acid.

Best would be a study of the specificity carried out on genuine pectic substances, but these are very difficult or impossible to obtain. The activity on some commercial pectins was tested in a series of experiments. In all cases the two fungi exhibited considerable enzyme activity (Table 28). No explanation can be given for the different levels of activity on these pectins as there was no obvious connection between the activity and the level of any of the components found in the pectins (Table 22), nor between the apple and citrus pectins.

Comparisons, made by iodometry of the reducing groups liberated, showed that the polygalacturonase of *S. sclerotiorum* exhibited high and almost equal activity on pectin and pectic acid (in agreement with Echandi & Walker 1957), and a greatly inhibitory effect of Ca^{++}-ions on the activity on pectin and nearly a nullification on the pectic acid (Figure 38). The polygalacturonase from *B. cinerea* was more active on pectic acid than on pectin, but the effect of the Ca^{++}-ions, though more pronounced, showed the same pattern (Figure 39) as found in the case of *S. sclerotiorum*. The different activity on pectin and pectic acid is in agreement with the results of Sherwood (1966) but it is in disagreement with those of Wood & Gupta (1958). The reason for this might lie in different proportions of the PG's known to be produced by *B. cinerea* (cf. the literature survey

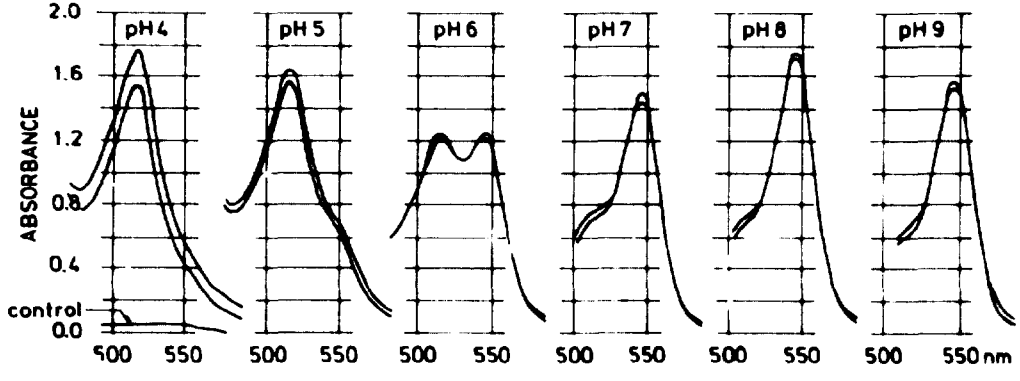

Figure 36. An example of the activity of *Botrytis cinerea* (No. 931) polygalacturonase (PG) and pectin lyase at different pH's measured as absorbance in the Unicam 800 double-beam spectrophotometer. The 515 nm is the maximum absorbance of the galacturonic acid unit released by the activity of the PG, and the 547 nm is the maximum absorbance of the $\Delta_{4:5}$-deoxy compound formed by the activity of the pectin lyase. Two replicates.

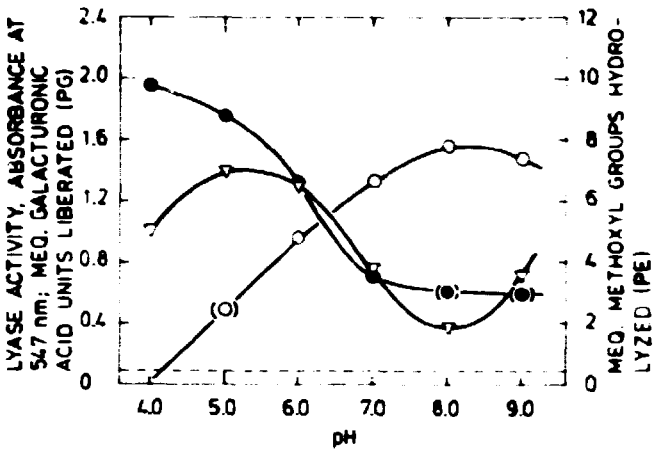

Figure 37. The activity at different pH's of *Botrytis cinerea* (No. 931) pectolytic enzymes measured simultaneously on pectin, compare Figure 36. ● give the polygalacturonase activity in micro-equivalents of the galacturonic acid units liberated. ○ give the absorbance at 347 nm of the $\Delta_{4:5}$-deoxy compounds formed by the pectin lyase. ▽ give the activity of pectinesterase as micro-equivalents of the methoxyl groups hydrolyzed. ( ) possibly too high values as the activity is calculated from a weak shoulder on the curve of absorbance for the other enzyme (cf. the method of calculation above).

Table 28. Pectolytic activity of culture filtrates from *Sclerotinia sclerotiorum* and *Botrytis cinerea* on some commercial pectins at pH 5.0.

Enzyme preparation from culture of	Commercial pectin used as substrate	Measured enzyme activity of		
		polygalacturonase	pectin lyase	pectinesterase
		galacturonic acid, meq per ml	4,5-deoxygalacturonic acid, absorbance	meq methyl groups hydrolyzed
S. sclerotiorum	Genu, citrus N.F.	1.91	0	7.25
	Fluka, citrus	1.29	0	5.40
	Fluka, apple	2.03	0	5.10
	BDH, apple	1.38	0	4.55
B. cinerea	Genu, citrus N.F.	0.67	0.34	1.80
	Fluka, citrus	0.51	0.34	1.30
	Fluka, apple	0.66	0.27	1.80
	BDH, apple	0.59	0.25	1.70

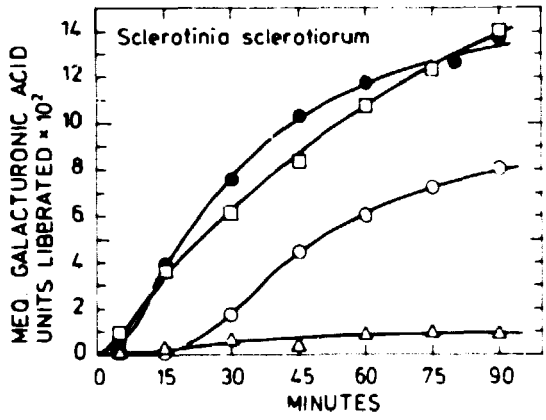

Figure 38. The effect of $CaCl_2$ on the rate of activity of polygalacturonase from *Sclerotinia sclerotiorum* (No. 794) on pectin and pectic acid solutions at pH 5 when measured by iodometry of the reducing groups released. ● : pectin without salt added; ○ : pectin + 0.054N $CaCl_2$; □ : pectic acid without salt added; △ : pectic acid + 0.054N $CaCl_2$ (0.027N $CaCl_2$ = 0.2% $CaCl_2,2H_2O$.

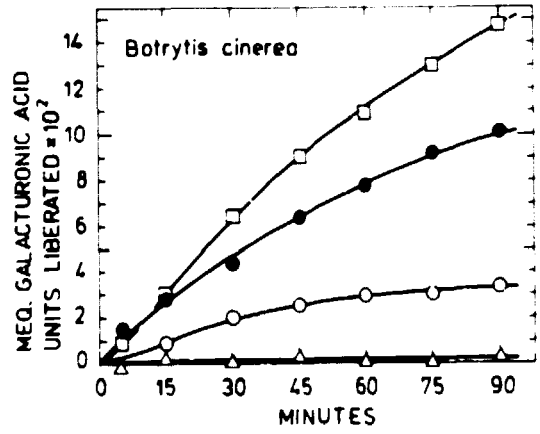

Figure 39. The effect of $CaCl_2$ on the rate of activity of polygalacturonase from *Botrytis cinerea* (No. 43) on pectin and pectic acid solutions at pH 5 when measured by iodometry of the reducing groups released. ● : pectin without salt added; ○ : pectin + 0.054N $CaCl_2$; □ : pectic acid without salt added; △ : pectic acid + 0.054N $CaCl_2$ (0.027N $CaCl_2$ = 0.2% $CaCl_2,2H_2O$).

in paragraph 6.2). The same high polygalacturonase activity on pectic acid and the strong calcium effect was further demonstrated by means of the TBA-method (Figure 40). Using this method it was also found that *S. sclerotiorum* infrequently exhibited a small pectin lyase activity on pectin, which was nearly only detectable because of the occurrence of the reaction product in the culture filtrates (Table 29), and, as was the case with *B. cinerea*, a relatively high degree of saponification occurred simultaneous to the lyase activity. In contrast, *B. cinerea* exhibited great pectin lyase activity on pectin and little on pectic acid. The activity of this enzyme was enhanced by small concentrations of calcium (cf. the review on the effect of calcium), and the activity was only weakly reduced by a further increase of the calcium concentration (Figures 33, 34 and 40).

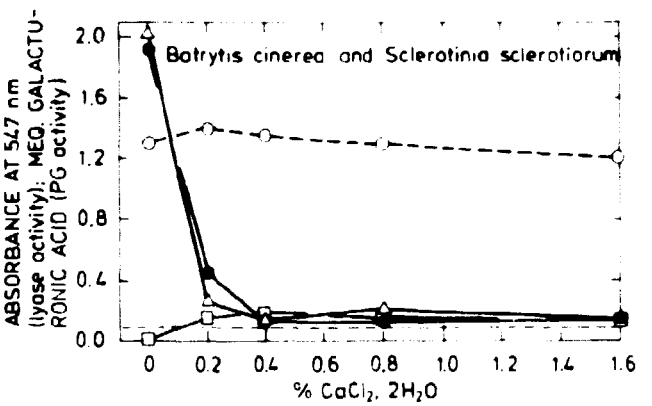

Figure 40. The effect of $CaCl_2$ on the activity of polygalacturonase (PG) and pectin lyase (PL) from *Botrytis cinerea*, and of PG from *Sclerotinia sclerotiorum* on solutions of pectin or pectic acid when measured as the galacturonic acid units and $\Delta^{4:5}$-deoxy compounds formed by the 2-thiobarbituric acid (TBA) method. ● : *B. cinerea* PG on pectic acid at pH 5.0; △ : *S. sclerotiorum* PG on pectic acid at pH 5.0; □ : *B. cinerea* PL on pectic acid at pH 8.5; –○–: *B. cinerea* PL on pectin at pH 8.5. Reactions below the thin dashed line are uncertain.

Table 29. Pectolytic activity of culture filtrate from *Sclerotinia sclerotiorum* at pH 5.0 and 8.5. Infrequently occurring results with both polygalacturonase (515nm) and lyase (547nm) activity, and simultaneous with this a relatively high degree of hydrolysis of methoxyl groups at pH 8.5.

Enzyme preparation from culture of	Activity at pH	Absorbance, nm				meq methoxyl groups produced
		515	547	515	547	
		reaction products in control		measured enzyme activity		
S. sclerotiorum No. 794	5.0	0.04	0.05	1.11	0	6.85
S. sclerotiorum No. 975	5.0	0.03	0.03	1.94	0	6.70
S. sclerotiorum No. 794	8.5	-	-	0.07	0.21	1.20
S. sclerotiorum No. 975	8.5	-	-	0.18	0.47	1.60

The inhibitory effect of calcium on the PG activity increases with the concentration, as shown both by iodometry and by the TBA-reaction, and it is not just a salt effect because equal concentrations of NaCl are without any effect (Figures 41 and 42).

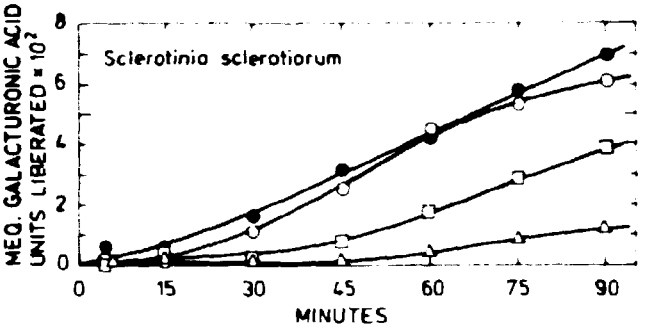

Figure 41. The effect of NaCl and $CaCl_2$ on the rate of activity of polygalacturonase from *Sclerotinia sclerotiorum* (No. 794) on pectin solution at pH 5.0 when measured by iodometry of the reducing groups released. ● : without salt added; □ : 0.027N $CaCl_2$; △ : 0.054N $CaCl_2$; ○ : 0.027N NaCl (0.027N $CaCl_2$ = 0.2% $CaCl_2,2H_2O$).

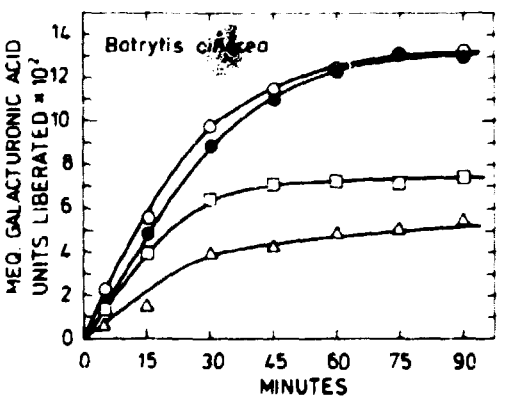

Figure 42. The effect of NaCl and $CaCl_2$ on the rate of activity of polygalacturonase from *Botrytis cinerea* (No. 43) on pectin solution at pH 5.0 when measured by iodometry of the reducing groups released. ●: without salt added; □: 0.027N $CaCl_2$; △: 0.054N $CaCl_2$; ○ : 0.027N NaCl (0.027N $CaCl_2$ = 0.2% $CaCl_2,2H_2O$).

Both *B. cinerea* and *S. sclerotiorum* produce relatively large amounts of oxalic acid during growth (this production will be discussed in detail in paragraph 8.3.3. below). The oxalic acid transferred with the culture filtrate to the reaction mixture did not influence the enzyme activity, as this did not change whether or not $CaCl_2$ was added in amounts equivalent to this oxalic acid (Figures 43-45). On the other hand, the oxalic acid may enhance the PG activity by removing Ca^{++}-ions from the solution in an environment where the enzyme is active, e.g., in carrot tissue. Figures 43-45 also show a direct comparison between the PG activities of the two fungi on pectin. Independent of the level of activity, the effect of Ca^{++}-ions was always greater on the *S. sclerotiorum* enzyme, i.e. the slope of the curve is much steeper, than on the *B. cinerea* enzyme. A simultaneous analysis for PE activity showed the same overall picture as discussed above (Figure 46), although it was less uniform, possibly due to titration at half-hour intervals during the three hours' reaction time instead of the continuous titration used when the PE activity was tested alone. In these cases there is a tendency that neutralization of the oxalic acid present or small calcium concentrations stimulate the enzymatic hydrolysis of the methoxyl groups.

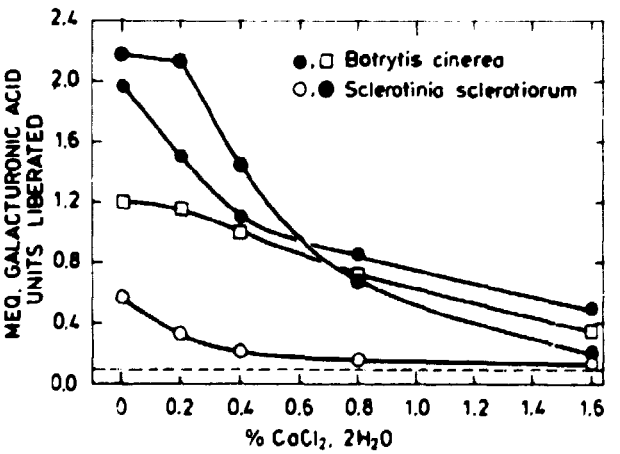

Figure 43. The effect of $CaCl_2$ at two levels of activity of polygalacturonase (culture filtrates) at pH 5.0 from *Botrytis cinerea* (No. 43) and *Sclerotinia sclerotiorum* (No. 794) on pectin solution as measured by the TBA method.

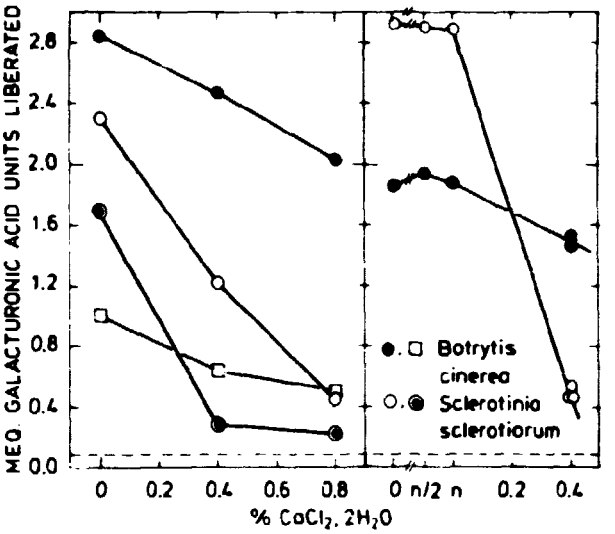

Figure 44. The effect of $CaCl_2$ at three levels (culture filtrates in two series of experiments) of activity of polygalacturonase at pH 5.0 from *Botrytis cinerea* (No. 43) and *Sclerotinia sclerotiorum* (No. 793 and 794) on pectin solution as measured by the TBA method. n/2 and n mean the addition of calcium in amounts half-equivalent and equivalent, respectively, to the oxalic acid occurring in the culture filtrates.

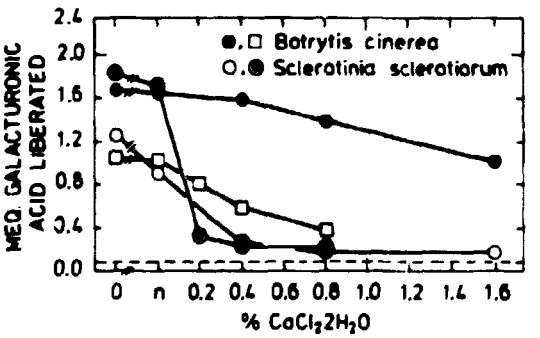

Figure 45. The effect of $CaCl_2$ at two levels of activity of polygalacturonase (culture filtrates) at pH 5.0 from *Botrytis cinerea* (No. 43 in half concentration, ● , and No. 931, □) and *Sclerotinia sclerotiorum* (No. 793, ○ , and No. 794, ◉) on pectin solution as measured by the TBA method. n means addition of calcium equivalent to the oxalic acid occurring in the culture filtrates.

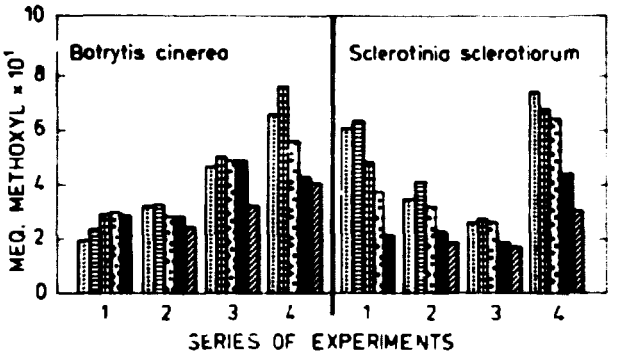

Figure 46. During the three-hour reaction time with polygalacturonase from culture filtrates of the fungi on pectin solutions, the reaction mixture was titrated frequently with 0.1N NaOH in order to keep it at pH 5.0. Here the total amounts of NaOH used are given as equivalents of hydrolyzed methoxyl groups for four series of experiments. They should be compared with the results of continuous titration presented in paragraph 8.3.1. ⊡ : without addition of $CaCl_2$; ⊟ : addition of $CaCl_2$ equivalent to the oxalic acid occurring in the reaction mixture; ⊞ : 0.014N $CaCl_2$; ⊡ : 0.027N $CaCl_2$; ▨ : 0.054N $CaCl_2$; ▨ : 0.108N $CaCl_2$ (0.027N $CaCl_2$ = 0.2% $CaCl_2,2H_2O$).

In addition to these experiments, three other carrot pathogens were tested for pectolytic activity after growth on carrots as well as on defined media.

During growth on the asparagine pectin medium, *Mycocentrospora acerina* accumulated considerable amounts of galacturonic acid and $\Delta_{4;5}$-deoxygalacturonic acid units in the medium, but on the pectin proper no activity of the causal enzymes could be detected at pH 5.0 and 8.5 at the conditions used, and only very weak hydrolysis of the methoxyl groups occurred. Tests with *Chalaropsis thielavioides* gave the same results, only with much less accumulation of reaction products in the culture medium (Table 30). Comparable results were obtained after growth on sterile carrot tissue. The only difference was that the juice

Table 30. Comparison of pectolytic activity from *Sclerotinia sclerotiorum*, *Mycocentrospora acerina*, and *Chalaropsis thielavioides* after growth on asparagine pectin medium.

Enzyme preparation from culture of	Activity at pH	Reaction products accumulated in growth medium		Measured enzyme activity of		
		galacturonic acid, per ml	$\Delta_{4,5}$-deoxy-galacturonic acid, absorbance at 547 nm	polygalacturonase	pectin lyase	pectinesterase
				galacturonic acid, meq per ml	$\Delta_{4,5}$-deoxy-galacturonic acid, absorbance	methoxyl groups hydrolyzed
S. sclerotiorum No. 794	5.0	0.54	0	0.88	0	5.00
S. sclerotiorum No. 975	5.0	0.38	0	1.07	0	3.65
M. acerina	5.0	0.92	0.44	0	0	0.30
C. thielavioides	5.0	0.19	0.15	0	0	0.25
S. sclerotiorum No. 794	8.5	-	-	0.15	0.03*	0.70
S. sclerotiorum No. 975	8.5	-	-	0.14	0	0.55
M. acerina	8.5	-	-	0.15	0	[illegible]
C. thielavioides	8.5	-	-	0	0.05	0.60

*doubtful.

from healthy carrots contained a substance that with TBA exhibited a maximum absorption at 525 nm. This substance obviously disappeared during the growth of all the fungi except *M. acerina* (Table 31). Under these conditions, the fresh carrot juice showed no enzyme activity but both *S. sclerotiorum* and *S. cinerea* produced the pectolytic enzymes on carrots as vigorously as on the defined media (Table 31), and the PG was affected in the same way by external calcium supplied to the reaction mixture (Figure 47).

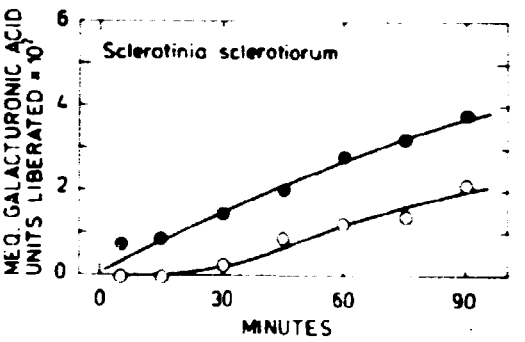

Figure 47. The effect of $CaCl_2$ on the activity of polygalacturonase from carrot juice attacked by *Sclerotinia sclerotiorum*. Measured by iodometry of the reducing groups liberated at pH 6.0. ●: activity on pectin solution; ○: activity on pectin solution with 0.054N $CaCl_2$ added.

8.3.3. Oxalic Acid

Significant amounts of oxalic acid accumulated in the growth medium were not observed until about the fourth day of growth, consequently it is regarded as unlikely that this compund contributes to the initial steps of the pathogenesis. However, in the later stages of the development of the rot, oxalic acid may contribute seriously to the acceleration of the decay.

From about the fourth to the tenth day of growth at room temperature, a very steep increase occurred in the production of oxalic acid by *S. sclerotiorum*. During prolonged growth of

Table 31. Enzyme activity in juice from fresh carrots and from carrots inoculated with various carrot pathogens.

Enzyme preparation from	Reaction products accumulated in growth medium			Measured enzyme activity of							
	515 nm	525 nm	547 nm	polygalacturonase			pectin lyase			pectinesterase	
	gal. acid, ml^{-1}	unknown substance	$\Delta_{4,5}$-deoxygal. acid, abs.	at pH 5.0			at pH 8.5			at pH 5.0	at pH 8.5
				515 nm	525 nm	547 nm	515 nm	525 nm	547 nm		
				gal. acid, meq per ml	unknown substance	$\Delta_{4,5}$-deoxygal. acid, abs.	gal. acid, meq per ml	unknown substance	$\Delta_{4,5}$-deoxygal. acid, abs.	methoxyl groups hydrolyzed	
Juice of fresh carrots	0	0.17	0	0	0	0	0	0	0	0	0.45
Juice of carrots inoculated with:											
S. sclerotiorum No. 794	0.13	0.04	0.04	1.34	0	0	0.13	0.04	0.04	5.65	0.50
S. sclerotiorum No. 975	0.13	0.04	0.04	1.10	0	0	trace	0	trace	3.25	0.60
B. cinerea No. 931	0.13	0.04	0.04	1.11	0	0	0.19	0	0.34	5.45	1.30
M. acerina	0	0.38	0	0	0	0	0	0	0	0	0.55
C. thielaviocides	0	0.09	0	0	0.09	0	0	0.09	0	0	0.80

the fungus no further increase in the accumulation of oxalic acid occurred (Figure 48). In *B. cinerea* the production of oxalic acid in the growth medium is much slower as no considerable amounts occur until about the twelfth day and later (Figure 48).

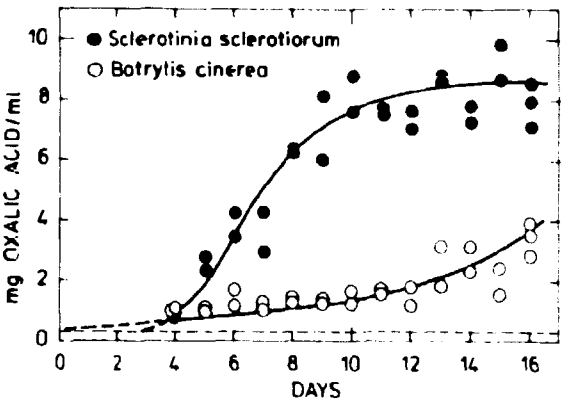

Figure 48. Formation of oxalic acid during the growth of the two fungi.

The production of oxalic acid is temperature dependent (Figure 49) in the same way as is the growth and the production of pectolytic enzymes.

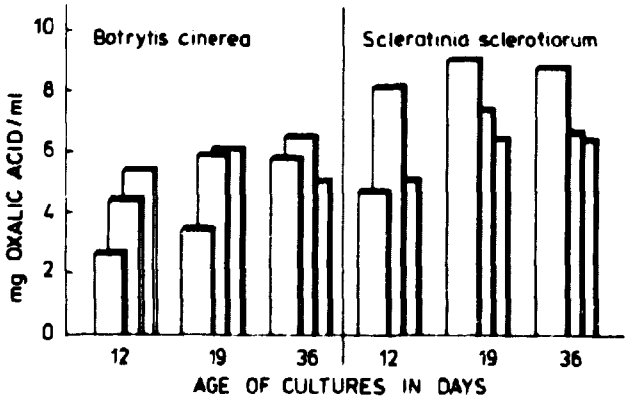

Figure 49. The effect of growth temperature on the formation of oxalic acid during the growth of the two fungi. Columns from left to right represent 15, 25 and 28°C, respectively.

The effect of great amounts of oxalic acid in the growth medium or in the later state of rotting is evident, but it does not tell much about the in situ production in plant tissue in close connection with the pectolytic enzymes, pectic substances and calcium. It is suggested that much smaller amounts of oxalic acid may have a pronounced effect as competitors to the free carboxylic groups that capture the calcium ions or remove them from the pectic substances of the middle lamellae.

Nothing in the experiments points to the oxalic acid affecting the pectolytic enzymes, apart from the fact that the substrate becomes more available.

8.4. Summary

The majority of the experiments dealt with the enzymes produced by *B. cinerea* and *S. sclerotiorum*, but supplementary experiments were made with *Chalaropsis thielavioides* and *Mycocentrospora acerina*, which cause black lesions and black crown rot (licorice rot) of carrots, respectively.

Pectinesterase (PE) produced by *B. cinerea* and *S. sclerotiorum* is not an adaptive enzyme in sensu stricto as indicated by others authors. It was easily demonstrated that the fungi produce PE without the presence of pectin, but also that PE production was strongly increased in the presence of pectin. There were great differences in the amounts of PE produced by different isolates of the fungi.

Glucose is a very poor carbon source for the production of pectolytic enzymes, whereas asparagine as second carbon source or nitrogen source induces or greatly stimulates the production. Calcium in the growth medium enhances the production or activity of both PE and polygalacturonase (PG) but hardly of the lyase.

PE activity is initially proportional with the reaction time. Later, the activity fades out and stops. Both Na^+- and Ca^{++}-ions stimulated PE activity, but there was a characteristic difference in the effect of the two cations. First, the stimulatory effect was considerably higher for Ca^{++} than for Na^+ at the same ion strength, but the reaction stopped at a lower level of hydrolysis. Secondly, although the Na^+ had a clearly stimulating effect, the reaction stopped at the same level of hydrolysis as did the reaction without addition of salts. The termination of enzyme activity was not related to exhausting

of the substrate, or inactivation of the enzyme, as a later addition of further enzyme did not result in a renewed increase of activity.

The curves express the PE activity proper, but under the influence of simultaneous action of PG or PMG on the molecular chain, which may influence the final result. The specific effect of the Ca^{++}-ions is regarded as cross-linkages between carboxylic groups along different molecular chains, or chain fractions, and this prevents parts of the methyl ester groups from hydrolysis proportional to the amount of calcium added. At the same time, the Ca^{++}-ions inhibit the PG activity and this may further influence the PE activity.

The PE had an optimum at pH 5.0, but activity or methyl ester hydrolysis was found at all the pH levels examined, lowest, however, at about pH 8.0 and with a secondary increase at pH 9.0. This may either be caused by saponification, or by another esterase active at the high pH level. If just a saponification, the culture filtrates from *B. cinerea* and *S. sclerotiorum* should behave similarly, but this secondary hydrolysis was always highest in the case of *B. cinerea*. In this connection it should be mentioned that hydrolysis at this high level of pH may be facilitated by the activity of pectin lyase in the presence of calcium, as there is a parallelism between their reaction curves. Therefore it cannot be left out of account that pectin lyase activity enhances the rate of this secondary methyl ester hydrolysis.

The PE activity of Fluka and Sigma commercial 'pectinases' clearly differs in behaviour from that of *B. cinerea* and *S. sclerotiorum*.

B. cinerea and *S. sclerotiorum* grow fairly well on galacturonic acid as the sole source of carbon, but the acid accumulated in media with pectin as source of carbon until it disappeared in the late growth of the fungi. Also the degradation product from lyase activity, $\Delta_{4:5}$-deoxygalacturonic acid, accumulates in the growth medium and disappears in the later growth period of the fungi. For this reason, it is probably metabolised like galacturonic acid.

The reaction products started to accumulate as soon as there was measurable growth, but at this time the amount of enzymes in the culture filtrates was so small that activity could not be

detected by the methods used.

The enzymes accumulate in the growth medium during growth and their activity is then measured in cell-free culture filtrates as the amount of degradation products, galacturonic acid or $\Delta_{4:5}$-deoxygalacturonic acid, liberated from the added pectin.

The method was modified to allow for simultaneous reaction and measurement of the activity. Under these conditions, which may approach those in nature, it was shown that PG had a pH optimum below 5.0, but that it still had a measurable activity at pH 9.0. The lyase had an optimum at about pH 8.0, but not much lower activity at pH 9.0, and no activity at pH 4.0. It is most essential to state that under these conditions all three enzymes exhibit high activity about pH 6.0, the normal level for fresh carrots, though it is not optimal for each of them.

Little has been written on the pectolytic activity on pectin and pectic acid of enzymes produced by *B. cinerea* and *S. sclerotiorum*. The present author found that, under the conditions used, PG from *B. cinerea* was more active on pectic acid than on pectin and that 0.054N Ca^{++}-ions nullified the activity on pectic acid but, although they were strongly inhibitory, they left some activity on the pectin. The enzyme of *S. sclerotiorum* showed equal activity on pectin and pectic acid, but the effect of calcium was almost the same as on the *B. cinerea* enzyme activity.

B. cinerea culture filtrates had a high pectin lyase activity on pectin and little on pectic acid, and it was confirmed that small concentrations of calcium enhanced the activity. To this, it should be added that increasing amounts of calcium in the reaction mixture only slightly reduced the lyase activity. Only infrequently could any lyase activity be detected from *S. sclerotiorum*, and then almost only as an accumulation of $\Delta_{4:5}$-deoxygalacturonic acid in the culture filtrate.

Both fungi produce relatively large amounts of oxalic acid during growth. Some of this acid is transferred to the reaction mixture, but it has no influence on the enzyme activity, which did not change upon removal of the oxalic acid by equivalent amounts of calcium. On the other hand, oxalic acid may enhance PG activity by removing Ca^{++}-ions from an environment where the enzyme is active, e.g., carrot tissue.

Direct comparison between the PG activities of the two fungi on pectin showed that the effect of the Ca^{++}-ions was always much

greater on the *S. sclerotiorum* enzyme than on that of *B. cinerea*.

No qualitative difference was found between the enzymes produced in culture or in carrot tissue by the two fungi. The carrot tissue itself exhibited a weak PE activity under the experimental conditions used and contained a substance that gave a colour reaction in the TBA test with absorption maximum between those of the degradation products of the PG and lyase activity.

Mycocentrospora acerina and *Chalaropsis thielavioides* accumulated galacturonic acid and $\Delta_{4:5}$-deoxygalacturonic acid when grown on artificial media and on carrot tissue, but the amount of enzyme in the culture filtrates was too small to give measurable reactions on pectin proper under the conditions used. Only a weak hydrolysis of the methoxyl groups was detected.

Analyses of the production of oxalic acid revealed no accumulation until about the fourth day of growth, for which reason this acid may not contribute much to the incipient stage of pathogenesis, unless small amounts have an effect in statu nascendi. At the later stage, however, oxalic acid accumulates so much that it may contribute heavily to the acceleration of the decay, because its removal of calcium enhances the activity of the polygalacturonase.

S. sclerotiorum produces a large amount of oxalic acid in the first ten days of growth, whereas *B. cinerea* is a slow producer of the acid.

The necessity of large amounts of oxalic acid for the additive or synergistic action together with polygalacturonase is doubtful, because small amounts in situ may compete with the free carboxylic groups to capture the free calcium ions, or remove them from the pectic substances of the middle lamellae.

9. APPENDIX

> The task of reviewing some more or less circumscribed portion of a rapidly expanding scientific subject like plant pathology never has been an easy one, and the passage of time does not make it any easier. Far from it - for year by year the flood of research reports from newly founded and from older much enlarged research institutes continues to rise; and even with the help of abstracting journals, it is becoming more and more difficult to keep in touch with the current output and to correlate it with the earlier work.
>
> William Brown 1965

9.1. General Review on Pectolytic Activity

The literature on pectolytic activity is very extensive and has increased almost explosively during the last two decades during which the present author has pursued his work. The following review is given partly with the aim to present and discuss the importance, uniformity and distribution of these enzymes in nature, and partly in order to give a general view in relation to the work presented above.

In order to facilitate comparison of the organisms discussed, recent generic names are used throughout the paper. The survey below gives these names together with the synonyms used by various authors (Table 32).

The eighth edition of Bergey's Manual of Determinative Bacteriology (Buchanan & Gibbons 1974) is followed for the names of soft-rot bacteria. The present author agrees with the comments on page 333 of this manual that discuss the difficulties with the intermediate strains, and that the degradation of pectic substances cannot be regarded a 'good character' for separating species because it runs across other important characters, and because degradation is not only an all-or-none reaction always involving just one and the same enzymatic process. However, the practical importance of these characters (sic) in applied plant pathology may not be left out of account.

Table 32. Recent generic names of the species and their synonyms as used by various authors. The table is included in order to facilitate the comparison of species otherwise disguised by different names.

Organisms	References
Fungi:	
Alternaria radicina Meier, Drechs. & Eddy syn. *Stemphylium radicinum* (Meier, Drechs. & Eddy) Neerg.	91
Alternaria solani Sor.	418
Aphanomyces euteiches Drechs.	21
Aspergillus carbonarius (Bainier) Thom syn. *A. fonsecaeus* Thom & Raper	3, 119, 120
Aspergillus niger v. Tiegh.	3, 7, 8, 9, 119, 127, 142, 146, 332, 345
Botryosphaeria ribis Gross. & Dug.	201, 246
Botrytis allii Munn	173
Botrytis cinerea Pers. ex Fr., st. asc. *Sclerotinia fuckeliana* (de By.) Fckl. (this fungus is one of the main objects of the present work; see also the review on page 102)	14, 16, 17, 37, 53, 54, 66, 67, 68, 69, 70, 80, 82, 83, 84, 94, 103, 104, 107, 123, 128, 133, 134, 141, 144, 145, 173, 211, 272, 344, 345, 364, 392, 398, 400, 401, 404, 418, 426
Botrytis fabae Sardiña	103
Botrytis ricini Godfrey, st. asc. *Sclerotinia ricini* Godfrey	402
Botrytis squamosa Walker, st. asc. *Sclerotinia squamosa* (Viennot-Bourgin) Dennis	173
Candida pseudotropicalis (Cast.) Basgal	240
Chalaropsis thielavioides Peyronel (included in some experiments in the present work)	16, 210

Table 32, continued

Organisms	References
Colletotrichum gloeosporioides (Penz.) Sacc. syn. *Gloeosporium kaki* Hori (one of very many synonyms) st. asc. *Glomerella cingulata* (Stonem.) Spauld. & Schrenk	221, 397
Colletotrichum lindemuthianum (Sacc.& Magn.) Briosi & Cav.	4, 130
Colletotrichum trifolii Bain & Essary	166, 172
Coniella diplodiella (Speg.) Petr. & Syd. syn. *Coniothyrium diplodiella* (Speg.) Sacc.	123, 124, 125, 126, 127, 128, 332, 394
Fusarium argillaceum (Fr.) Sacc., st. asc. *Nectriopsis solani* (Reinke & Berth.) Booth, syn. *Hypomyces solani* Reinke & Berth. f. sp. *cucurbitae* Snyd. & Hans.	170, 171, 174
Fusarium avenaceum (Cda. ex Fr.) Sacc. syn. *F. roseum* (Link) Snyd. & Hans., 'Avenaceum' f. sp. *cerealis* (Cke.), st. asc. *Gibberella avenacea* Cke.	282
Fusarium moniliforme Sheldon, st. asc. *Gibberella fujikuroi* (Saw.) Wr.	69, 347
Fusarium oxysporum Schlecht. ex Fr. + *formae specialis*	57, 86, 87, 88, 111, 116, 117, 135, 154, 261, 284, 304, 327, 344, 345, 394, 413, 418
Fusarium solani (Mart.) Sacc. + *formae specialis*	36, 118, 168, 331, 418
Geotrichum candidum Link ex Pers.	26
Helicosporium mompa Tanaka	37
Kluyveromyces fragilis (Jørg.) v.d. Walt, syn. *Saccharomyces fragilis* Jørg.	99, 240, 312
Mycocentrospora acerina (Hartig) Deighton syn. *Centrospora acerina* (Hartig) Newhall (included in some experiments in the present work)	16, 210

Table 32, continued

Organisms	References
Nectria radicicola Gerlach & Nilsson st. conid. *Cylindrocarpon destructans* (Zins.) Scholten, syn. *C. radicicola* Wr.	118
Penicillium chrysogenum Thom	119, 311
Penicillium expansum Link emend. Thom	83, 84, 372, 395
Phoma betae (Oud.) Frank, st. asc. *Pleospora bjoerlingii* Byford	73, 74
Phoma medicaginis Malbr. & Roum syn. *Ascochyta imperfecta* Pk.	172
Phytophthora cryptogea Pethyb. & Laff.	121
Potebniamyces discolor (Mout. & Sacc.) Smerlis, st. conid. *Phaecidiopycnis furfuracea* (Rostr.) Jørst., syn. *Pyrenochaeta furfuracea* (Fr.) Rostr.	83
Pyrenochaeta terrestris (Hans.) Gorenz	198, 214
Pythium debaryanum Hesse	17, 67, 68, 70, 94, 133, 418
Rhizoctonia praticola Kotila, st. basid. *Corticium praticola* Kotila	164, 305
Rhizoctonia solani Kühn, st. basid. *Thanatephorus cucumeris* (Frank) Donk	22, 33, 34, 35, 37, 42, 43, 100, 118, 230, 238, 305, 344, 345
Rhizopus stolonifer (Ehr. ex Fr.) Link, syn. *R. nigricans* Ehr.	30, 344, 345, 371
Rhizopus tritici Saitô	70, 179, 332, 434
Sclerotinia fructigena Aderh. & Ruhl., syn. *Monilinia fructigena* (Aderh. & Ruhl.) Honey, st. conid. *Monilia fructigena* Pers.	77, 78, 79, 82, 83, 84, 199
Sclerotinia laxa (Aderh. & Ruhl.) Worm., syn. *Monilinia laxa* (Aderh. & Ruhl.) Honey, st. conid. *Monilia cinerea* Bon.	77, 82
Sclerotinia sclerotiorum (Lib.) de By., syn. *S. libertiana* Fckl., *Whetzeliana sclerotiorum* (Lib.) Korf & Dumont	16, 29, 53, 54, 72, 113, 123, 128, 165, 167, 169, 223, 224,

Table 32, continued

Organisms	References
(this fungus is one of the main objects of the present work; see also the review on page 97)	242, 243, 263, 264, 276, 278, 300, 322, 400, 401, 410, 420, 421
Sclerotinia trifoliorum Eriks.	185, 322, 420, 421
Sclerotium rolfsii Sacc., st. basid. *Corticium rolfsii* Curzi	39, 41, 344, 345
Stemphylium botryosum Wallr., st. asc. *Pleospora herbarum* (Pers. ex Fr.) Rabenh.	172
Thielaviopsis basicola (Berk. & Br.) Ferraris	244
Verticillium albo-atrum Reinke & Berth.	30, 86, 98, 182, 213, 283, 284, 285, 309, 396, 417
Verticillium dahliae Kleb.	320
Verticillium nigrescens Pethyb.	417
Bacteria:	
Aeromonas liquefaciens (Beijer.) Kluyver & van Niel	71, 200
Arthrobacter 547	331
Bacillus megaterium de By.	133, 134, 333
Bacillus polymyxa (Prazmowski) Mig.	12, 186, 286, 287, 288, 331, 332, 365, 366, 386, 387
Bacillus subtilis Cohn	12, 133, 134, 331, 333, 365, 366
Clostridium aurantibutyricum Hellinger	186
Clostridium butyricum Prazmowski, syn. *Bacillus amylobacter* v. Tiegh.	186, 393
Clostridium felsineum (Carbone & Tombolato) Bergey et al.	12, 20, 40, 186, 397
Clostridium multifermentans Bergey et al.	252, 253, 254, 271
Clostridium tertium (Henry) Bergey et al. - probably identical with *C. pectinivorum* (Störmer) Donker	186, 393

Table 32, continued

Organisms	References
Erwinia carotovora (Jones) Bergey et al., syn. *Pectobacterium carotovorum* (Jones) Waldee	49, 50, 71, 76, 133, 134, 280, 365, 366, 386, 387, 389, 390, 404, 409
Erwinia carotovora (Jones) Bergey et al. var. *carotovora* (Jones) Dye, syn. *E. aroideae* (Town.) Bergey et al.	59, 70, 96, 113, 121, 186, 331, 384, 385, 422, 426
Erwinia carotovora (Jones) Bergey et al. var. *atroseptica* (van Hall) Dye, syn. *E. atroseptica* (van Hall) Jennison, *E. phytophthora* (Appel) Bergey et al., *Pectobacterium carotovorum* (Jones) Waldee var. *atrosepticum* (van Hall) Hellmers & Dows.	59, 136, 163, 164, 331, 367, 383, 384, 385, 425
Erwinia chrysanthemi Burkh. et al., syn. *Pectobacterium carotovorum* (Jones) Waldee var. *chrysanthemi* (Burkh. et al.) Graham & Dows., *P. partheni* (Starr) Hellmers, *P. partheni* (Starr) Hellmers var. *dianthicola* Hellmers	30, 31, 32, 59, 140, 143, 187, 321, 332, 365, 366, 384, 385
Pseudomonas marginalis (Brown) Stevens	138, 291
Pseudomonas solanacearum (E.F. Sm.) E.F. Sm.	418
Pseudomonas syringae van Hall	365, 366
Pseudomonas spp. and various other bacteria	314, 315, 331, 332, 365, 366
Rhizobium spp.	296
Rumen bacteria	155
<u>Higher plants</u>:	
Beans (*Phaseolus vulgaris* L.)	243
Carrots (*Daucus carota* L.)	180, 262, 301, 302, 303, 332
Cranberry (*Oxycoccus macrocarpon* (Ait.) Pursh. syn. *Vaccinium macrocarpon* Ait.)	15
Cucumber (*Cucumis sativus* L.)	45

Table 32, continued

Organisms	References
Oats (*Avena sativa* L.)	205
Onions (*Allium cepa* L.)	370
Oranges (*Citrus spp.*)	203, 249, 369
Peas (*Pisum sativum* L.)	7
Tomato (*Lycopersicum esculentum* Mill.)	33, 246, 247, 248, 262, 270, 302, 303, 307, 332, 338
Various legumes	236, 296
Animals:	
Snails (*Helix pomita* L. and others)	194, 195, 262
Insects (*Pyrrhocoris apterus* L. and others)	44, 89
Protozoa (the rumen ciliate *Ophryoscolex purkynei* Stein, and others)	44, 254
Phytonematodes	110
Earth-worms	262

9.1.1. Fungi

Concerning the authors of the organisms, see Table 32.

Alternaria radicina. Two isolates of *A. radicina* from carrots have produced PG adaptively and PE in culture The PG was more active on polypectate than on pectin and with a clear difference between the two isolates (Curren 1969). In the present author's experiments an old isolate of the fungus failed to produce pectolytic enzymes both on plants and in culture.

Alternaria solani. As discussed above under *B. cinerea*, *A. solani* only exhibits weak PE and PG activities as compared with those from *B. cinerea*, 7 *formae speciales* of *Fusarium oxysporum*, 2 *f.sp.* of *F. solani*, and *Pseudomonas solanacearum* (Winstead & Walker 1954). However, the conditions cannot be regarded as optimal as these authors used pH 7 for PE and pH 4 for PG determinations.

Aphanomyces euteiches. The fungus has been tested for production of pectolytic enzymes after growth on pea seedlings (*Pisum sativum* L.) as well as on complex or synthetic media (Ayers & Papavizas 1965). Both in vivo and in vitro *A. euteiches* produces an extracellular pectic acid depolymerase (i.e. an endo-PG according to Demain & Phaff (1957) as cited by others). It is a constitutive enzyme reducing the viscosity of Na-polypectate faster than pectin, and it has been shown that the resistance against the enzyme increases with the degree of methoxylation of the pectinic acid. The molecular chain was randomly degraded into smaller units with lower viscosity, but without the accumulation of significant amounts of mono- and di-galacturonic acid. The optimum pH was 8 for Na-polypectate and 9 for pectinic acid in 0.02M tris-HCl buffer. These pH values are surprisingly high for this type of enzyme compared to the PG's produced by other organisms. Perhaps the optimum pH 9 for the degradation of pectinic acid is a result of interaction with the saponification of the methoxyl groups.

No PE was produced in vitro, but in infected pea seedlings the activity was greater than in non-inoculated healthy plants. It cannot be disregarded that the growth of the fungus has caused a release of host PE from the cell walls as this enzyme is more

easily extracted by the NaCl-solution, cf. Ayers & Papavizas 1965, but no tests were made to determine whether all the PE was of host origin. Normally the PE of plant origin has a higher pH optimum than those produced by fungi (cf. paragraph 9.1.3.).

No lyase was detected, and as a further indication of its absence, the addition of 10^{-3}M $CaCl_2$ or 10^{-3}M EDTA was without effect on the reactions.

Aspergillus carbonarius. Edstrom & Phaff (1964a) tested the activity of pectin lyase of *A. carbonarius* together with 7 other species or strains of *Aspergillus* and with *Penicillium chrysogenum*. The fungi were grown on 2% pectin as carbon source, and after 24 hours the UV optical density at 235 nm, specific for pectin lyase, was measured in a reaction mixture containing the culture fluid. All the *Aspergillii* produced pectin lyase but in very different amounts with *A. carbonarius* as the most active followed by a mutant of *A. niger*, while under these conditions *P. chrysogenum* exhibited no lyase activity. Besides the production of lyase, *A. carbonarius* produced considerable amounts of PE and PG, the latter with 5-6 times the activity of the lyase produced in the same course of time. The purified lyase (see above) has a plateau of activity about pH 5-6 and a peak at pH 8.5 in the presence of 0.04M tris-acetate buffer. The activity of the enzyme increased with the cation concentration, least with sodium, somewhat more with magnesium, and most with calcium, which showed a surprisingly high optimum concentration of 0.13M $CaCl_2$ in comparison with other lyases that have an optimal requirement for calcium of about 10^{-3}M (cf. Table 17).

The pectin lyase from *A. carbonarius* mainly split pectin into unsaturated methyl esters of tri-, tetra-, and pentagalacturonic acid together with a smaller amount of the di-ester and only traces of longer chain pieces. In experiments using oligogalacturonate esters it was shown that the enzyme could not act on the trimer and lower esters (Edstrom & Phaff 1964b).

Aspergillus niger. This fungus has a pronounced ability to degrade pectic substances, and it is known as a soft-rot-causing organism second to none. The enzyme production was first studied by Gäumann & Böhni (1947b), who found a production of pectinase, i.e. PG, and pectase, i.e. PE, the former of which

may or may not be stimulated in the presence of pectin, while the latter was only produced in the presence of pectin and somewhat inhibited in the presence of glucose together with the pectin in the growth media. On this basis, the authors concluded that, in *A. niger*, PG is a constitutive and PE an adaptive enzyme, respectively, as was also found to be the case with *Botrytis cinerea* (Gäumann & Böhni 1947a). During growth there was first a drop in pH and then a rise to a level higher than the initial pH. This may be substrate-dependent and of importance for the enzyme production.

A. niger also produces an exo-PG according to Saito (1954, in Endo 1964), compare the discussion of *Coniella diplodiella* below.

The first detection of lyase activity on pectic substances was obviously made by Albersheim, Neukom & Deuel (1960a) using the commercial enzyme preparation Pectasin R-10 (apparently the same as Pectinol R-10 and originating from *A. niger*, cf. Albersheim & Killias 1962, table 1). In many respects this lyase behaves like that of *A. carbonarius* (cf. e.g. Albersheim 1966). Albersheim & Killias (1962) used pH 5.2 in their experiments as it was found optimal for the purified pectin lyase (Albersheim, Neukom & Deuel 1960), but this may depend on the salt concentration (Edstrom & Phaff 1964a), as may be the apparent effect of the different buffers. Under these conditions Albersheim & Killias (1962) found that calcium had an inhibitory effect on pectin degradation. This is true but not without modifications. According to several other authors, the pectin or pectate lyases are calcium dependent with an optimal concentration of about 10^{-3}M above which an inhibition sets in (cf. Table 17). However, this is not inconsistent with the figures given by Albersheim & Killias (1962) when put together on a graph (Figure 50) where the dashed part of the curve starts at the activity comparable to that of unbuffered medium at pH 5.2 (zero activity normally only occurring when EDTA is added in order to remove traces of calcium, compare e.g. Preiss & Ashwell 1963), and where the course of the curve shows optimum at a very low calcium concentration. The effect of higher concentrations of calcium on the activity of the lyase follows the same pattern as on the PG activity and may possibly be an effect on the pectin rather than on the enzyme. Further, Albersheim & Killias

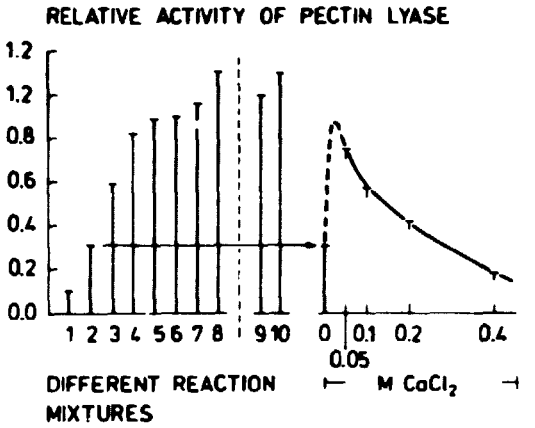

Figure 50. Relative activity of pectin lyase from *Aspergillus niger* (Pectinol R-10) in different buffers and concentrations of $CaCl_2$. (Drawn after Albersheim & Killias 1962, Tables 2 and 3, and calculated to the same scale). 1: no buffer, pH 4.0; 2: no buffer, pH 5.2. All other reaction mixtures are buffered with 0.1M concentrations at pH 5.2; 3: Na-acetate; 4: K-citrate; 5: Na-citrate; 6: Na-phosphate; 7: K-phosphate; 8: McIlvaine's buffer. In cases to the right of the dashed line, phosphate-citrate buffer is used, though with no buffer added, inserted at zero concentration of $CaCl_2$; 9: phosphate-citrate buffer control; 10: 0.4M NaCl.

(1962) studied the effect of several buffers. The activity was lowest without buffer. It was fairly high in citrate buffer, high in phosphate buffer, and highest in McIlvaine's buffer, all in 0.1M concentration. But all these buffers interact with the calcium ion and disturb or obscure its effect. These effects should be seen in connection with the statements of Albersheim (1966) that polyvalent anions, such as phosphate and citrate buffers, enhance the lyase activity to twice the magnitude in acetate buffer - which is surprising because phosphate and citrate buffers are inhibitors of the calcium effect, or vice versa, and as calcium is generally an absolute requirement for lyase activity. On the other hand, calcium enhances the elimina-

tive cleavage of 68% esterified pectin in acetate or tris-acetate buffers, but not the rate of cleavage of fully methyl-esterified galacturonates (Edstrom & Phaff 1964a).

The pectin lyase of *A. niger* is of the endo-type such as that of *A. carbonarius* (Albersheim 1966).

The enzyme production of *A. niger* was studied on a glucose-Na-polypectate-salts medium and on a pectin-salt medium by Sherwood (1966), who used the supernatant from centrifugation as enzyme preparation after adjusting to the desired pH. He found the lyase activity of *A. niger* more pronounced on Na-polypectate than on pectin and more active at pH 7.2 than at 5.0. This may perhaps explain why some of the *A. niger* strains in the experiments of Edstrom & Phaff (1964a) only exhibited low lyase activity on a pectin-containing reaction mixture. However, there may be other interactions affecting the lyase activity. Among the reaction products of heat-treated pectin buffered to pH 6.8 were terminally located $\Delta_{4:5}$-unsaturated uronic acids, which fact leads to the supposition that small amounts of these compounds are present in commercial pectins. Such pectins are inhibitory to pectin lyase, just as in the case of $\Delta_{4:5}$-deoxygalacturonic acid as a reaction product of the lyase activity (Albersheim, Neukom & Deuel 1960b, Albersheim 1963).

A. niger produces a PG that is more active on Na-polypectate than on pectin at pH 5.0 (Sherwood 1966) and on exo-PG with the same behaviour (Saito 1954, quoted from Endo 1964). Saito was obviously the first who obtained an exo-PG from the complex of pectolytic enzymes. His preparations were free of endo-PG but exhibited PE activity.

Up until now, *A. niger* has been stated to produce three endo-PG's, two endo-PMG's, two exo-PG's, and one endo-PL besides pectinesterase (Koller 1966, Rombouts & Pilnik 1972). This constitution forms the basis for the pronounced ability of *A. niger* to disintegrate plant tissues under a variety of conditions, pathogenic as well as saprophytic.

Finally, it should be added that *A. niger* causes crown rot in peanut seedlings with necrosis, which is apparently induced by oxalic acid (Gibson 1953).

Botryosphaeria ribis. *B. ribis* is a canker-forming invader of woody tissue. It was used for studies of the maceration of

plant tissues in comparison with commercial 'pectinase' and enzyme preparations from tomato fruits (McClendon & Somers 1960). Enzyme preparations were made from apples rotted by *B. ribis* and from fresh tomato fruits extracted with 10% NaCl. The ultrafiltered extracts or solutions were used for the experiments on slices of different storage organs of plants, mainly potato and turnip. The commercial 'pectinase' and the preparation from *B. ribis* had maximum macerating ability at pH 3.0-3.5, whereas that from tomatoes was most active at a pH of about 4.7. Calcium ions did not prevent maceration although they increased the firmness of the tissue, and EDTA could not itself macerate the tissues but it facilitated the enzymatic maceration. The reason for this may be a removal of calcium from the tissue.

Below pH 3.0, a non-enzymatic maceration developed rapidly.

It should be added that the high production of cellulase by *B. ribis* might support the pectolytic activity during pathogenesis (cf. Husain & Kelman 1959).

Botrytis ricini. *B. ricini* causes browning and maceration of castor-bean (*Ricinus communis* L.) pericarps. Thomas & Orellana (1964) stated that adaptively produced pectolytic and cellulytic enzymes play an important role in these processes. In susceptible varieties, more water-soluble pectin was present than in the resistant varieties. At the same time the pericarp of resistant castor-beans contains much more calcium and magnesium and far less sodium and potassium than does the pericarp of susceptible beans, in which the proportions were the opposite with a very high potassium content.

Candida pseudotropicalis. Pectolytic activity seems uncommon in *Endomycetes* and *Blastomycetes* (asporogenous yeasts) according to the investigations of Luh & Phaff (1951) who searched for this property in 181 species and strains and only found it present in *Kluyveromyces fragilis* (syn. *Saccharomyces fragilis*) and in *C. pseudotropicalis* (considered by Diddens & Lodder 1939 to be the imperfect state of *K. fragilis*). The two fungi cannot use pectin as a carbon source. The activity results from the effect of a constitutive exocellular enzyme with PG-like properties, but it causes only partial hydrolysis without forming galacturonic acid. The enzyme had an optimal pH of 3.5-4.0 and a

maximal rate of activity at 55-60°C, which is higher than for other known pectolytic enzymes. The fungi did not produce PE.

Demain & Phaff (1954) studied the pectolytic enzyme of *K. fragilis* in greater detail and found evidence of two linear phases in the breakdown of pectic acid. The first phase, lasting for 30 min., was characterized by a rapid liberation of reducing groups leading to about 25% hydrolysis, at which a mixture of penta-, tetra-, tri- and digalacturonic acid accumulated. In the second phase, lasting for about two hours, these smaller units of pectic acid were degraded in different ways and some of the degradation products were inhibitory for further hydrolysis. Digalacturonic acid was not attacked. On this basis the authors concluded that the yeast PG requires at least two neighbouring free carboxyl groups to permit hydrolysis of the corresponding linkage. Later, Phaff (1966) stated the enzyme to be an endo-PG hydrolyzing polygalacturonides containing three or more galacturonic acid units. In the present author's opinion, the two-phase reaction still renders it probable that more than one enzyme is involved (compare, e.g., Starr & Chatterjie 1972).

Colletotrichum gloeosporioides, investigated under the name *Gloeosporium kaki*, one of many synonyms for the conidial stage of *Glomerella cingulata*. The relations between the soft-rot caused by this fungus and the pectolytic enzymes produced by the host (*Diospyros kaki* L., i.e. persimmons or date-plums) were studied by Tani (1967). Crude enzyme preparations were made by salting out with $(NH_4)_2SO_4$ from healthy and diseased fruits and by culture filtrates, which were dialysed against water, fractioned and used for the assays. pH was maintained by McIlvaine's citrate-phosphate buffer and the activity was measured by conventional methods.

The macerating activity was greatest at pH 4.5-5.0 and greater on the kaki fruits than on potato tissue.

Endo-PMG (optimum pH 5), endo-PG (optimum pH 6), exo-PG (optimum pH 5), and PE were present. The pH optimum for PE is not given and this enzyme was not detected in culture filtrates. Pectin and pectate lyases could not be detected in the same preparations as the above enzymes. As none of the fractions, after fractionating the enzymes, had the ability to cause soft-rot in kaki fruits, or to macerate the fruit tissue, the author

concluded that *C. gloeosporioides* was not alone responsible for the soft-rot.

From the healthy fruit tissue, Tani (1967) isolated two macerating components with little activity on pectin and pectic acid. However, microscopic examinations showed that they caused separation of the cells along the middle lamellae, and their activity caused a disappearance of soluble pectin and pectic acid and a reduction in the insoluble fraction of pectic substances. These facts led Tani (1967) to suggest that the two components might be special pectolytic enzymes resembling those produced by *Clostridium felsineum*, which is a very active bacterium in the wet retting process used on flax (see the discussion of this below).

When kaki fruits were attacked by *C. gloeosporioides* more of the two components could be isolated than from healthy fruits. The differences between the fungal enzymes and those from the fruits were very pronounced as, e.g., 2000 endo-PMG units/ml of *C. gloeosporioides* culture filtrate were required to macerate the fruit tissue, while the fruit enzymes macerated the fruit tissue at concentrations that had little or no activity on pectin or pectic acid.

It is possible that the fungal enzymes enhance the liberation of the fruit-tissue enzymes from the cell walls. Thus, this secondary effect is the primary cause of the soft-rot of the kaki fruits.

Parallel to this is the softening of kaki fruits as a result of gamma radiation (Kitagawa, Yamane & Iawata 1964). Further, Tani (1967) drew attention to the note of Wood (1960) on the possibility that plant tissues differ greatly in susceptibility to attacks by macerating enzymes. He offered definite examples in his paper and supplemented them with results of analogous studies with *B. cinerea*, of which a virulent isolate showed three kinds of tissue-specific macerating enzyme activities (cf. Tani & Nanba 1969).

Colletotrichum lindemuthianum. Though pectolytic enzymes from various microorganisms were found to degrade pectic substances in almost the same manner, their biochemical properties may be rather different. Hence, e.g. Albersheim & Anderson (1971) extracted proteins from the cell walls of bean hypocotyls, tomato

stems and suspension-cultured sycamore cells that completely inhibited the activity of polygalacturonases secreted from *C. lindemuthianum*, *F. oxysporum* and *S. rolfsii*. The purified inhibitor of *C. lindemuthianum* PG was 40 times more effective on this enzyme than on the PG from *F. oxysporum* and no effect could be demonstrated on *S. rolfsii* PG. The purified bean hypocotyl protein combines with the *C. lindemuthianum* PG to form a complex with a dissociation constant of 10^{-9}M or less, which means that it acts as an agglutinine. English et al. (1972) found that *C. lindemuthianum* produces an endo-PG that prefers polygalacturonic acid to pectin as substrate, and reduces the viscosity of a solution at optimum pH 5.0 by 50% for a 1% hydrolysis of the glycosidic linkages, though the initial as well as the terminal products were tri- and di-galacturonic acids with minor amounts of the monomer. This endo-PG not only removed up to 75% of the galacturonic acid units from the cell walls, but also small amounts of the sugars. The fungus exudates several polysaccharide-degrading enzymes during growth. 'Pectinase', however, exudates and reaches maximum activity first (English, Jurale & Albersheim 1971).

Colletotrichum trifolii. The pectolytic activities of *C. trifolii*, *Stemphylium botryosum* and *Phoma medicaginis* (syn. *Ascochyta imperfecta*) were compared by Hancock & Millar (1965). The fungi produced neither endo-PG nor endo-PMG in vitro, nor did they attack lucerne tissues. However, they produced exo-PG in vitro but not in vivo. *C. trifolii* and *S. botryosum* produced PE on autoclaved lucerne stems, while *P. medicaginis* produced very little or no PE in vitro and in vivo. There was a strong increase in the PE activity during attack by *C. trifolii*. Such an increase was not connected with the attack by *S. botryosum*. Though the three fungi produced lyase on the autoclaved lucerne stems, only *C. trifolii* produced considerable lyase activity during the attack. This was confirmed by a pronounced decrease in methoxyl groups of the pectins in the lesions caused by *C. trifolii*, but not in those caused by the other two fungi. On this basis the authors suggested that of these three fungi only the pectolytic enzymes play a role in the pathogenesis of *C. trifolii*.

The characteristics of the *C. trifolii* pectate lyase were

further investigated by Hancock (1966b). During growth of the fungus on autoclaved lucerne stems, the pH drifted from 6 to 9 and only in the last part of this decrease in H^+-activity was the lyase production considerable (compare the discussion under *B. cinerea*). In a liquid medium containing pectic acid as carbon source, the lyase production of the culture filtrate followed the decrease in H^+-activity and not the mycelial growth, which itself contained only very little lyase activity. The exo-PG production was parallel to the decrease in H^+-activity and could be detected 5-7 days earlier than the lyase. These results could lead to the supposition that the lyase is only liberated when the hyphae undergo lysis, but it was indicated that it is in fact an exo-cellular enzyme.

Coniella diplodiella. *C. diplodiella* is a very strong producer of pectolytic enzymes as compared to *B. cinerea* and *S. sclerotiorum* (Endo 1961a, b, Endo & Miura 1961). The fungus produces three different endo-PG's, an exo-PG and PE (Endo 1961a, 1963a, b, 1964, Swinburne & Corden 1967, Rombouts & Pilnik 1972). The endo-PG's were considerably more active in hydrolyzing pectic acid than in pectin, and they hydrolized 3, 4 and 10% of the glycosidic linkages for 50% reduction in viscosity at the optimal pH of 4.0 to 4.5. The exo-PG had the same pH optimum. Pectin and pectinic acid were little affected by this enzyme (Endo 1964).

Fusarium species. The activity of pectate lyase from *F. argillaceum* was found to be calcium-dependent (Hancock & Stanghellini 1968) like other pectolytic lyases. Pectate lyase activity could be completely inhibited by EDTA in the absence of an exogenous supply of Ca^{++}. By testing the essentiality of Ca^{++} for the lyase activity in a semi-solid medium, it was found that the optimal concentration lies above 10^{-3}M, and that high concentrations of calcium proved inhibitory to the pectate breakdown. When the molar ratio of uronic acid/Ca^{++} is decreased towards 2 in the reaction mixture, the enzymatic breakdown is enhanced and at a ratio lower than 2 the reaction rate is reduced, possibly because of gel formation. In this connection, Hancock & Stanghellini (1968) showed the Ca^{++} concentration to be higher in the lesions caused by *F. argillaceum* than in the adjacent healthy tissue of the squash plant, and by ^{45}Ca autoradiography

they found that calcium accumulated in the middle lamellae of the infected tissue. However, they also found that this accumulation of calcium around the lesions did not greatly influence the rate of maceration of the tissue by the pectate lyase. The amount of pectate lyase varied greatly with the strain and the culture medium used (Hancock, Eldridge & Alexander 1970) and it was produced in different physical forms too (Hancock 1976).

Brown & Wood (1953) found the production of 'pectinase' in *F. moniliforme* highly adaptive and Singh & Wood (1956) added that the fungus secreted macerating enzymes in liquid media only when natural extracts, pectic substances or galacturonic acid were present.

With asparagine as nitrogen source, the enzyme production was higher than with peptone or nitrate.

For the same macerating effect the loss of viscosity of pectin was considerably less for *F. moniliforme* than for *E. carotovora* var. *carotovora*, indicating that the enzyme of the former was "more exo" than the latter. The enzymes of *F. moniliforme* were more active on high methoxyl pectin than on pectate when measured as the liberation of reducing groups.

Several formae specialis of *Fusarium oxysporum* and *F. solani* were compared with some other organisms regarding the production and nature of pectolytic enzymes by Winstead & Walker (1954, see the discussion under *B. cinerea*). The *Fusaria* produced only traces of a PG on replacement cultures and very little in wheat bran. Under both sets of conditions there was a considerable production of PE. The enzymes extracted from such wheat-bran cultures of *F. oxysporum f.sp. lycopersici* caused vascular browning and wilt in the same way as the attack by the fungus (Gothoskar et al. 1955). These facts raise the question of whether the effect would be maintained with purified enzymes or with PE free from other pectolytic enzymes. It was demonstrated that the PE could not itself cause browning or wilting, though the authors found a convincing correlation between the severity of vascular browning and the PE activity, which they could not explain. However, the results revealed the PG responsible for the effect. This enzyme was further identified as a depolymerase (i.e. endo-PG) because it did not produce mono-galacturonic acid during the action. The authors suggest that the symptoms originate partly from the plugging of the

vessels by degradation products from the enzymatic activity and partly from liberated phenolic compounds.

The reason why the vascular browning is correlated with the PE activity is perhaps already revealed in the results of Waggoner & Dimond (1955), who found the PG (endo-PG) of *F. oxysporum f.sp. lycopersici* more active on pectin in the presence of PE and that PE in the absence of PG was unable to reduce the viscosity of pectin or to macerate tomato stem tissue. If this PG - like many other PG's - is more active on de-esterified pectin than on the pectin itself, the consequence will be, all things considered, that the PG activity enhances with increasing activity of PE. Waggoner & Dimond (1955) also found that the amount of PG and PE was maximal on the third day of growth, after which the PG decreased more rapidly than the PE. This supports the findings of high PE activity together with a low PG activity. The pH changed in accordance with the enzyme activity as it dropped from the initial value of 5 to 4 and then rose steadily until it levelled out at pH 8. The PE from healthy tomato stem tissue was much more active at pH 7 than at pH 5, while the fungal PE was equally active at the two pH's. No PG could be detected in the healthy plant tissue. Finally, Waggoner & Dimond (1955) formulated the hypothesis that calcium uronide gels are formed - as an effect of the enzymatic action - and that these lodge in the vessels and obstruct the flow and thus cause a water shortage. This contributes to the wilting symptoms at the same time as the maceration liberates phenolic glycosides, which become hydrolyzed by fungal enzymes. Then the phenolic compounds oxidize and produce the discoloration.

Bloom & Walker (1955) did not find any effect on the wilting caused by *F. oxysporum f.sp. lycopersici* when they sprayed tomato plants with $CaCl_2$ solutions. This, however, may be a question of possible uptake in the tissue (cf. the investigations of the present author). Later, Edington & Walker (1958) inoculated the fungus on tomato plants grown in media with levels of calcium varying from 5 to 500 ppm. They found a progressive decrease in attacks with serious wilting symptoms with increasing calcium content. The results of Cordon (1965) are in agreement with these observations as he found increased attack by the fungus when the tomato plants became deficient in Ca^{++} after infection, but less if the deficiency occurred before infection.

Ca^{++}-ions inhibited the activity of the F. *oxysporum* f.sp. *lycopersici* PG; apparently because it interferes with the growth of the fungus or with the availability of the pectic substances. Increasing Ca^{++} concentration in the vascular sap reduced the disease index, and the Ca^{++}-induced reduction of the PG activity was linear in a double-logarithmic scale. This invalidates the hypothesis of Waggoner & Dimond (1955). The calcium could not, however, bring the degradation to a complete stop. These results should be compared with the experiments of the present author.

The NAA (α-naphthalenacetic acid) induced resistance against F. *oxysporum* f.sp. *lycopersici* increased markedly with increasing supply of calcium to the plants. This may be due to a higher calcium content of the pectic substances, as in fact they become more water-insoluble, and the hydrolysis of the pectic substances in tissues treated either with NAA or calcium was slower than that in untreated plant tissue (Corden & Edington 1960, Edington, Corden & Dimond 1961).

In susceptible, but not in resistant, tomato cultivars the fungus produced a PG activity that was correlated with the attack and development of symptoms, for which reason Mussell & Green (1970) concluded that the enzyme is of importance in the pathogenesis of F. *oxysporum* f.sp. *lycopersici*. F. *oxysporum* produced PG but no lyase on Na-pectate and pectin, whereas both enzymes were produced on autoclaved tomato stems. There was a weak PG activity and traces of lyase activity in extracts from healthy tomato stems. Both enhance considerably after inoculation with the fungus. Under these conditions, the PG and the lyase activity were almost equally high, which fact was ascribed to a high PE activity in the plants attacked (cf. Matta & Dimond 1963, Ferraris, Garibaldi & Matta 1974).

Retig & Lisker (1975) found increased activity of PG and lyase after inoculation in both susceptible and resistant tomato plants, but only the resistant plants remained symptomless. Hence the presence of cell-wall-degrading enzymes does not necessarily give rise to disease symptoms.

Cooper & Wood (1975) grew F. *oxysporum* f.sp. *lycopersici* and *Verticillium albo-atrum* on an inorganic salts medium with tomato stem cell walls and in different assay media with restricted carbon supply for studying the regulation and syn-

thesis of several cell-wall-degrading enzymes, the activities of which were determined by refined methods. Both fungi produced endo-PG and endo-lyase adaptively in the presence of galacturonic acid in concentrations not in excess of that required for growth, as higher concentrations repress the synthesis. Waggoner & Dimond (1955) found the PE of *F. oxysporum f.sp. lycopersici* produced constitutively.

Though the two chain splitting enzymes are of the endo-type, they may to a slight degree act in an exo-manner, because the first degradation products were the monomer and the dimer. It is suggested that the induction starts in this way. In the cell-wall cultures of *V. albo-atrum*, the endo-PG and the endo-lyase did not accumulate at the same time, which should be expected as galacturonic acid is inducer of both. Possibly this accumulation depends on the pH, which is optimum for the synthesis of endo-PG at about 5.5 and for endo-lyase at 7.5-8.0 (compare Bateman 1966, Hancock & Millar 1965, Hancock 1968). Sherwood's (1966) experiments included *F. oxysporum f.sp. lycopersici*, which he found to produce PG and pectin lyase with the same properties as those of *B. cinerea* and *Rhizoctonia solani* (compare the discussion under these fungi).

The PG may consist of several components as indicated for *F. oxysporum f.sp. lycopersici* and *C. diplodiella*. This may be due to differences in molecular size or to combination with other molecules. A highly developed method of purification and separation is necessary for determining the importance of this plurality (Swinburne & Corden 1967).

Drysdale & Langcake (1973) investigated the response of tomato plants to infection by *F. oxysporum f.sp. lycopersici* and gave in this connection a short review of some other papers dealing with the pectolytic enzymes produced by this fungus and of those produced by the plant too. From this it appears that PG is not present in the healthy stems and leaves of tomato though it is a normal constituent of the fruit. Further, that PE is present in the healthy plants and that its activity increases in the attacked plants. These findings support those of Waggoner & Dimond (1955). Drysdale & Langcake were able to distinguish between fungal PE and plant PE. The former had a broad pH optimum with the same activity at pH 7.0 and 5.5. That of plant origin had twice the activity at pH 7.0 of that at pH 5.5. A

1.5% concentration of the detergent Na-lauryl sulphate stopped the activity of plant PE while that from the fungus was only reduced by 40%. The two enzymes could be separated on DEAE-cellulose columns because of different adsorption capacity. Further, the fungal PE could be eluted from the infected plant by 0.025M NaCl while that from the plant was eluted by 0.5M NaCl.

Using these methods for distinguishing the PE's, the authors found that the fungal enzyme was only responsible for 5-10% of the PE activity in the infected plant. However, the investigations did not explain how the attack or the fungal culture filtrate stimulate the activity of the plant PE.

Using F. *oxysporum* f.sp. *cubense* as test organism, Page (1961) developed a quantitative chromatographic technique for the assay of PG activity in microcultures of single-spore isolates. At pH 3.7 the fungus hydrolyzed polygalacturonic acid to monogalacturonic acid, hence the enzyme should be of the exo-type.

Fusarium avenaceum produces an endo-pectate lyase in dry-rotted potatoes as well as in culture. The enzyme, crude as well as purified, causes a maceration and cell death of potato tissue (Mullen & Bateman 1971). With an optimum pH of 9.0-9.5 and an absolute requirement for Ca^{++}-ions, it behaves similarly to the lyases of several other *Fusaria*. The enzyme activity decreased in calcium concentrations above 0.9×10^{-3}M $CaCl_2$, and, in contrast, the endo-pectate lyase from F. *avenaceum* did not lose activity upon gel filtration in Sephadex 75, DEAE-cellulose chromatography and electrofocusing, which may indicate that the enzyme binds calcium ions as in the case of the lyases from *Erwinia carotovora* (Mount, Bateman & Basham 1970) and *E. chrysanthemi* (Garibaldi & Bateman 1971). See the discussion of the pectolytic activity of these organisms. Like other Ca^{++}-dependent lyases, it was inhibited by EDTA.

F. solani f.sp. *cucurbitae*. The pectolytic activity during attacks on squash (*Cucurbita maxima* Doch.) was studied by Hancock (1968), who found PE activity to be twice as high in the attacked tissue as in the healthy tissue, and that the PE could only be extracted from the healthy tissue when supplied wih NaCl, which also proved necessary for the activity of PE of plant as well as of fungal origin. This is uncommon for the latter type

of PE, and on studying Hancock's paper, the present author cannot see any reason why all the PE could not be of plant origin because the pectolytic activity during the attack may have liberated PE from the plant cell walls (compare, e.g., Tani 1967). Neither does it solve the problem that the fungus produces PE in artificial media. In spite of no increase in the specific PE in the tissue, the methoxyl content was found to be reduced by 47-73%.

The basic hydrolysis is primarily found to liberate methoxyl groups from the molecular chain; however, Hancock (1968) claimed that an alkaline depolymerization set in already with pH 7.7 at room temperature. During attack by *F. solani f.sp. cucurbitae*, the pH increased from 6.2 in healthy squash tissue to 7.4-7.7 in the diseased tissue, which facilitates the activity of the endo-pectate lyase found to be the responsible degrading enzyme produced by the fungus. The enzyme degraded Na-polypectate 4-5 times more rapidly than the degradation of pectin when determined by viscosimetry at pH 7.5. 90.5% esterified pectin (methylated polygalacturonic acid) could not be degraded by partially purified lyase unless PE activity was also present. On the other hand, preparations from culture degraded pectin more rapidly than pectate in the presence of 10^{-4}M Ca^{++}-ions at pH 8.5.

Bateman (1966) studied the pectolytic activity of *F. solani f.sp. phaseoli* after growth on artificial media, or on bean hypocotyls, either using the filtrated extracts directly, or after gel filtration on Sephadex G 75. The results showed that the fungus produces a PG degrading pectic acid (optimum pH near 6), a PMG more active on pectin than on pectate (optimum pH near 5), and a lyase that degrades both pectin and pectate under alkaline conditions with an optimum pH at or above 8.6. This enzyme is calcium-dependent with an optimal concentration near 1.7×10^{-2}M. Bateman (1966) mentioned that a calcium concentration above 10^{-3}M in mixtures containing 0.1% pectic acid caused a gelling and a subsequent decrease in the reaction rate. The enzyme is stated to be an endo-pectin lyase, which type is said to have no absolute requirement for calcium although this ion may stimulate the activity. It is therefore regarded as exceptional that the endo-pectin lyase of *F. solani f.sp. phaseoli* requires calcium (Rombouts 1972). There was no evidence that *F. solani f.sp.*

phaseoli produces PE.

The synergistic effect of *F. solani* and *R. solani* when their enzymes act together (Elarosi 1958) is discussed under the latter fungus.

Geotrichum candidum. Barash & Eyal (1970) found a PG produced by *G. candidum* that causes soft-rot of *Citrus*. The enzyme degraded pectic acid in a random hydrolysis in acetate buffer at the optimal pH 5. However, mono-galacturonic acid was observed immediately after the start of the reaction and it accumulated linearly until 40% hydrolysis, when 5% of the total galacturonic acid was liberated. Further hydrolysis resulted in a relatively faster accumulation of liberated galacturonic acid. Trigalacturonic acid was slowly hydrolyzed to mono- and digalacturonic acid. The latter was not further degraded. The authors concluded on this basis that the enzyme is an endo-PG.

Penicillium species. In *P. chrysogenum* both PG and PE are produced adaptively induced by pectin and pectic acid, and by galactonic, galacturonic, and mucic acid, all five having the same configuration in the last five C-atoms in the single units and molecules. The adaptive property is not absolute as very small amounts of the enzymes were produced without the presence of inducer (Phaff 1947). *P. chrysogenum* is also discussed under *Aspergillus carbonarius* (Edstrom & Phaff 1964a).

The behaviour of the pectolytic enzymes of *Penicillium expansum* was found to differ from that of *Sclerotinia fructigena*, *Polyporus versicolor* and *Botrytis cinerea* by Cole & Wood (1961a, b), as mentioned under the discussion of *B. cinerea*. These authors also found that *P. expansum* produces large amounts of galacturonic acid and short chain polymers of this acid on the hydrolysis of pectic substances due to the endo-PG and endo-PMG produced. Swinburne & Corden (1969) agree in these findings. They found, however, great differences between the pectolytic enzymes produced in vivo and in vitro by *P. expansum* when analysed in a crude state.

The crude, in-vitro-produced PG practically only liberated galacturonic acid during 24 hours of reaction time, in which the hydrolysis increased to 92%; during the same time the crude, in-vivo-produced PG liberated only little galacturonic acid, but

considerable amounts of trimer, tetramer, and higher polymers in which the hydrolysis reached a level of 52%. After this the enzymes should be of exo- and endo-type, respectively. It is therefore surprising that both enzymes showed identical endo-PG properties upon purification. There is still no explanation of this phenomenon but it might perhaps be related to the dissociation and recombination phenomena of components of PG, as discussed by Swinburne and Corden (1967). At least these results show how necessary it is to be cautious if applying conclusions drawn from in vitro tests to in vivo tests.

Besides the PG's, *P. expansum* produces a pectin lyase both in vitro and in vivo (Spalding & Abdul-Baki 1973). In vitro the fungus produces high amounts of the enzyme on pectin-polypectate medium, fairly high amounts on malic acid or citric acid, but nothing at all in the presence of glucose. Crude extracts from apples rotted by *P. expansum* contained pectin lyase as well as PE and PG. In the purified state this particular pectin lyase had an optimal activity at pH 6.5 in 0.4% citrus pectin. The activity decreased in higher pectin concentrations. Maceration and cellular death caused by this pectin lyase developed simultaneously and, as other pectin lyases, the enzyme is dependent on Ca^{++}-ions or other divalent cations (Figure 51).

Phoma betae. Crude extracts from healthy beets (*Beta vulgaris* L.) or from beets attacked by *P. betae* were used for studies of the activity of fungal enzymes in the tissues (Bugbee 1972, 1975). The fungus was found to produce exo-PG and endo-pectate lyase but no PE. Both enzymes were present in the decayed sugar-beet tissue, but only the endo-pectate lyase was present in the 3 mm adjacent to the rotted areas. The endo-pectate lyase exhibited different activities on pith, stem and root tissues with the highest activity on the pith and least on the root tissue (cf. Wood 1960, Tani 1967). Further, the lyase production was higher in susceptible than in less susceptible sugar beet cultivars. The activity of the enzyme strongly increased in the presence of 10^{-3}M $CaCl_2$, but not in 10^{-4}-10^{-8}M $CaCl_2$.

Phoma medicaginis is discussed under *Colletotrichum trifolii* above.

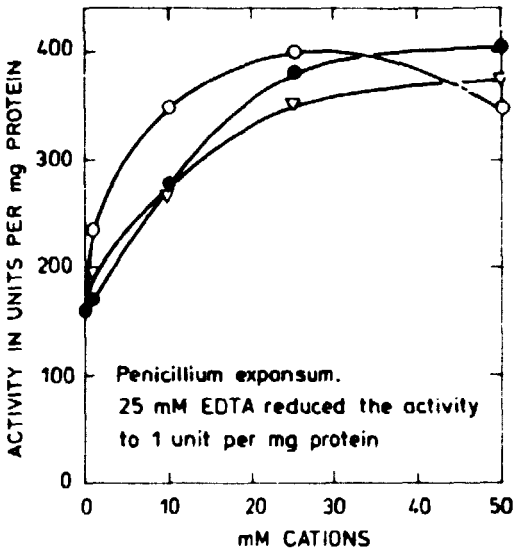

Figure 51. The stimulatory effect of divalent cations on the activity of pectin lyase from *Penicillium expansum*. ● : Ca^{++}-ions; ○: Mn^{++}-ions; ▽: Mg^{++}-ions. (Drawn after Spalding & Abdul-Baki 1973, Table 2).

Phytophthora cryptogea. P. *cryptogea*, causing dry rot, and *Erwinia carotovora* var. *carotovora* causing soft-rot of cucumber (*Cucumis sativus* L.) and summer squash (*Cucurbita pepo* L.) exhibit a synergistic pectolytic effect (El-Goorani, Abo-El-Dahab & Khosnnow 1976). After centrifugation of culture filtrates or of squash tissue extracts the supernatants were used for the assays, and the activity was determined by conventional methods.

The synergistic effect holds both for the PE activity and for the pectin-lyase activity, and both culture filtrates and extracts from diseased plants gave the same result. The cellulase activity was studied simultaneously in the same series of experiments, but it exhibited no synergistic effect.

A comparable synergistic effect was found with *Fusarium solani* and *Rhizoctonia solani* by Elarcsi (1958), who thought it possible that the synergism is brought about in the following manner:

pectin $\xrightarrow{\text{enzyme } \alpha}$ polyuronides $\xrightarrow{\text{enzyme } \beta}$ galacturonic acid.

If this is correct, a synergistic effect should be found in many other cases by using organisms with different patterns of pectolytic enzymes or even by the simultaneous action of host and parasite enzymes (see, e.g., the discussion of the *Colletotrichum gloeosporioides* attack on the kaki fruit, Tani 1967).

Pyrenochaeta terrestris. Pectin induces *P. terrestris* to produce an endo-PG but the production was repressed when the medium was supplemented by less than one gramme (0.05M) of glucose per litre (Horton & Keen 1966). On this basis the sugar content of onions was regarded as a factor in the resistance against pink rot (*P. terrestris*). Endo-PG and PE were both produced either in cultures or in onions. The purified enzyme preparations catalyzed at random hydrolyses of Na-polypectate faster than of oligouronides or pectin with an optimum for the reaction at pH 5. Therefore, the PG is listed as an endo-pectate form. Experiments with maceration of potato tissue showed a correlation with the activity of the endo-PG but not with that of PE. No lyase activity was detected (Keen & Horten 1966).

Pythium debaryanum. The pectolytic activity of this fungus has not been studied very extensively. It seems always to have been studied in comparison with other pathogens, mostly *B. cinerea* and *E. carotovora* (see under the discussion of these organisms), and this gives some valuable information.

Brown (1936, 1955) made a comparative study of the three organisms in question regarding their ability to macerate potato tissue. Conidia or enzymes of *B. cinerea* did not attack normal subturgid tissue, while cells or mycelium as well as the enzymes of *P. debaryanum* and *E. carotovora* attacked such tissue. Turgid tissue was attacked by all three organisms and their enzymes. Fernando (1937) compared the three organisms with *Bacillus subtilis*. Apart from his results with different growth media, his main results showed that *B. cinerea* had an acid optimum for pectolytic activity of about pH 6, that of *P. debaryanum* and *E. carotovora var. carotovora* was on the alkaline side at about pH 8, and that of *B. subtilis* between 7 and 9. Brown & Wood (1953) thought it probable that the 'pectinase' production in *P. debaryanum* and *E. carotovora var. carotovora* was constitutive, as

it can be produced on glucose as the sole carbon source, while in *Fusarium moniliforme* they found it to be adaptively induced by pectic substances and galacturonic acid. Ashour (1954) found that pectin does not increase the 'pectinase' production of *P. debaryanum*. This observation supports the findings of Brown & Wood (1953). In the same series of experiments Ashour (1954) found that pectin promotes 'pectinase' production of *B. cinerea*; this is in agreement with the findings of Gäumann & Böhni (1947a) as discussed under *B. cinerea*.

The results of Wood & Gupta (1958) with *P. debaryanum*, *B. cinerea* and *E. carotovora var. carotovora* are discussed under *B. cinerea*.

Rhizoctonia solani. Elarosi (1958) observed that the growth of *R. solani* was feeble on a medium with pectin as the sole carbon source compared to that of *Fusarium solani*. In the vicinity of a *F. solani* colony, growth of *R. solani* was stimulated. Mixed enzymes and extracts from double infection with the two fungi gave increased maceration and viscosity reduction compared to the activity from the two fungi separately. Such a synergistic effect was also found for *F. solani* and *Nectria radicicola* but not for *R. solani* and *N. radicicola*.

Crude enzymes and enzymes purified by ethanol precipitation were used for the test. Separation by paper chromatography of these preparations gave two bands from *R. solani* that had a stimulatory effect on the *F. solani* enzyme, while *F. solani* gave only one band that stimulated *R. solani*. Further, *R. solani* degraded pectin to galacturonic acid without detectable amounts of intermediates, while *F. solani* only degraded it to an intermediary state. On the basis of these investigations, it was concluded that the enzymes of *R. solani* and *N. radicicola* are of the same nature and different from those of *F. solani*, and a hypothesis was set forth on how the synergistic effect operates during pathogenesis (compare the case of synergism studied by El-Goorani, Abo-El-Dahab & Khoshnow (1976) discussed above).

These synergistic effects are important for understanding how different pectolytic enzymes may act together during pathogenesis.

The properties of protopectinase (the macerating enzyme) were also studied by Deshpande (1959), who found that the en-

zyme killed the cells before maceration was completed. Dilution or autoclaving reduced the macerating activity more than the toxicity, which demonstrated the presence of more than one component in the culture filtrate. Both the maceration and the toxicity had an optimum pH at 3-4 and at 7.4 when undialyzed, but at 3.5 and 6.8 after dialysis. Mono- and divalent inorganic salts reduced the enzyme activity, especially at higher concentrations. Divalent cations were most inhibitory, principally the Ca^{++}-ions.

Bateman (1963a, b) used dialyzed culture filtrates or extracts from diseased bean hypocotyls for the assay of enzyme activities of *R. solani*. Extracts from healthy plant tissue served as controls. Obviously, the fungus produced much more PG in culture than in the attacked plant tissue. The PG from culture degraded Na-polypectate to galacturonic acid. The PG from the diseased tissue degraded Na-polypectate to three different oligomers. Extraction of healthy and diseased tissue with 0.5M NaCl greatly increased the amount of plant PE, and the addition of NaCl to the dialyzed preparations strongly increased the activity of this enzyme, while salt was without effect on the activity of the fungal PE. In later experiments, Bateman (1964) found that water extracts from bean hypocotyls and macerated carrot tissue contained a dialysable inhibitor of PG. The inhibitor was found to be multivalent ions, especially Ca^{++}. Hydrolysis of the pectate was inhibited by Ba^{++} and Ca^{++}, but not by Mg^{++} or monovalent cations. The finding that bean hypocotyl tissue attacked by *R. solani* was more difficult to macerate with PG than was the healthy tissue, is supposed to be connected with the accumulation of Ca^{++}-ions in the tissue adjacent to the lesions. Bateman (1964) stated that the accumulation of Ca^{++} in the surroundings of the lesions resulted in a liberation of PE from the plant cell walls, and that pectic substances demethylated in the neighbourhood and ahead of the pathogen. The pectic substances thus demethylated then form Ca-pectates that are more or less inaccessible to PG. The pectin in the middle lamellae was detected by the ruthenium red method and the accumulation of Ca^{++} by ^{45}Ca autoradiography.

The hydrolysis of Ca-pectate may take place in the presence of oxalic acid, which is not produced by *R. solani*.

Preceding the investigations of the calcium effect, Bateman

& Lumsden (1965) found a simultaneous decrease in hydrolysable methoxyl groups of the pectin and an increase in the calcium content during growth and maturation of the bean hypocotyl. At the same time, the resistance increased against attacks by *R. solani*, as shown by a decrease in disease index. The nature of the pectin changes to pectinic acid; the free carboxyl groups of this acid form bridges with the calcium and make the molecules less accessible for degradation by PG's.

Thirty single-spore isolates (basidic spores) each of *R. solani* and *R. praticola* were tested for their ability to produce PG and PE (Papavizas & Ayers 1965). There was a pronounced difference between the two species regarding the production of these enzymes, which in both cases were produced adaptively. The single-spore isolates of both *R. solani* and *R. praticola* differed greatly in their ability to produce PG and PE. Fifteen isolates of each species produced neither PG nor PE. No relation was found between the pectolytic activity in vivo or in vitro and the virulence and the macerating ability of the isolates. Most of the single-spore isolates had a lesser virulence than their ancestors. Papavizas & Ayers (1965) were unable to detect lyase activity with certainty because their heated control gave a positive lyase reaction, but some isolates apparently produced lyase adaptively. However, Sherwood (1964, 1966) revealed the production of lyase by *R. solani*. The enzyme rapidly depolymerized pectin, somewhat more slowly the Na-polypectate. The PG from *R. solani* depolymerized Na-polypectate rapidly. The optimum pH was 8.2 for the lyase as against 4.2-4.6 for the PG by viscosimetry or 3.5 by maceration of potato tissue. Maceration by the culture filtrate was most intense at pH 5.0-6.5. Attention should here be drawn to the present author's investigations, which show that both PG and PE as well as lyase are active in this interval.

The relative activities of undialyzed, dialyzed, and dialyzed + 10^{-3}M $CaCl_2$ PG preparations were 11.2, 9.2, and 2.4, respectively. Those of corresponding pectin lyase preparations were 6.3, 3.3, and 2.9. Though the decrease in activity was much smaller for the lyase than for the PG, the results show that the activity lost by dialysis was not restored by adding 10^{-3}M $CaCl_2$. EDTA slightly inhibited the activity of dialyzed preparations so calcium and EDTA did not activate or inhibit the pectin lyase.

activity of *R. solani*. In Sherwood's (1966) experiments, the *R. solani* isolates did not produce PE. This is not in agreement with the findings of Bateman (1963a, b, 1967), but perhaps with those of Papavizas & Ayers (1965) as they found isolates without PE production.

Pectate lyase and PG from *R. solani* in culture filtrates and from attacked bean hypocotyl extracts were assayed after dialysis against distilled water. Extracts from healthy tissue served as control.

The pectate lyase had an optimum activity at pH 8 and the PG at pH 5, but both enzymes had so broad an activity range that some activity of the one enzyme was still measurable at the optimum pH of the other. This should be compared with the investigations of Ayers, Papavizas & Diem (1966) and of the present author.

$CaCl_2$ inhibited the activity of the PG but stimulated the pectate lyase. EDTA could stop the pectate lyase activity completely, but 10^{-3}M $CaCl_2$ clearly enhanced the activity to a level above that without addition of $CaCl_2$. The optimum was found to lie between 10^{-4} and 5×10^{-4}M $CaCl_2$. The latter concentration did not exhibit any effect on the PG activity, but increasing concentrations resulted in a progressive inhibition of the activity. Both pectate lyase and PG were produced adaptively (cf. Papavizas & Ayers 1965).

There was great variation between the enzyme activities of the isolates tested (Ayers, Papavizas & Diem 1966). One isolate did not produce the two enzymes. Five isolates showed a tendency that, when one was a weak producer of the one enzyme, it would also be a weak producer of the other, and vice versa. Compare the line drawn on the basis of the figures of these authors (Figure 52). One isolate produced a large quantity of PG and at the same time a little pectate lyase.

Bateman (1967) states that, in many cases, it is without doubt that cell-wall-degrading enzymes play a role for the establishment of the pathogen in the host, but there is now clear evidence that the ability to produce these enzymes in itself is insufficient to render a fungus pathogenic to plants.

In further studies with *R. solani*, Bateman (1967) used culture filtrates dialyzed against distilled water, and strained, centrifuged and dialyzed water, or NaCl extracts of diseased

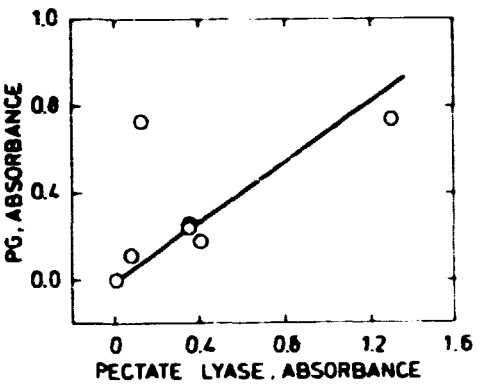

Figure 52. Comparison of the activity of polygalacturonase (PG) with that of pectate lyase from different isolates of *Rhizoctonia solani*. (Drawn after Ayers, Paravizas & Diem 1966, Table 2).

bean hypocotyls. The *R. solani* PE from culture filtrates remained unaffected by NaCl, while PE from diseased bean hypocotyls was inhibited by dialysis. This inhibition was removed by the addition of NaCl. The reason for this difference was that the main part of the PE from the diseased hypocotyls originated from the host (compare Drysdale & Langcake 1973). *R. solani* produces PE adaptively (in agreement with Papavizas & Ayers 1965).

Bateman (1967) stated that PE is always present prior to, during and after pathogenesis, and that the amount increases in the diseased tissue during pathogenesis. This author also suggested that PE has good conditions for activity in the bean hypocotyls because the pH is about 6 and does not change much during development of the disease. At this pH there is also considerable activity of pectate lyase and PG (cf. Bateman 1964, Bateman & Lumsden 1965).

Yamazaki (1957, quoted from Bateman 1967) found evidence that PE and PG act together during pathogenesis by *Helicobasidium mompa* on sweet potato (*Ipomoea batatas* (L.) Lam.). Endo (1965, quoted from Bateman 1967) stated that PE activity is necessary before endo-PG can clarify apple juice. On the basis of all these facts, Bateman (1967) states it to be most likely that PE acts synergistically with endo-PG during pathogenesis by *R. solani*, and that the PE in advance of the pathogen and its PG may result in

the formation of calcium pectate resistant to PG (cf. Elarosi 1958, Bateman 1964, Bateman & Lumsden 1965, El-Goorani, Abo-El-Dahab & Khoshnow 1976).

The amount of PG and lyase varies in the culture filtrates of *R. solani* depending upon the culture age (Bateman 1967). As the cultures grew old, the hydrolytic activity ceased but the lyase activity remained high (cf. Lisker, Katan & Henis 1975). There was more di-, tri- and tetra-galacturonic acid than mono-galacturonic acid in the reaction products of the diseased tissue; and as healthy tissue did not contain the enzymes, the author supposed that these products resulted from fungal activity, and hence that endo-PG was the only hydrolytic enzyme of greater importance beside the lyase in the diseased tissue.

R. solani produces several enzymes in vivo and in vitro that degrade various constituents of the cell walls. Investigations of these enzymes (Bateman et al. 1969) revealed that hemicellulases such as arabinase, xylanase and galacturonase were of apparent importance in the pathogenesis. The cell walls of young (4-day-old) susceptible bean seedlings could be degraded by *R. solani* enzymes, but not the cell walls of older plants (20-day-old) that were resistant to the pathogen (cf. the importance of calcium-pectate formation discussed above). The reason for this increase in resistance was not elucidated in the experiments made by Bateman et al. (1969), but it may have something to do with the chemical composition of the cell walls, such as for example the existence of cross-linkages among the cell-wall polymers. Perhaps proteins cross-linked by metal cations are also included in this structure (Ginzburg 1961).

Lisker, Katan & Henis (1975) studied the sequential production in *R. solani* of PG, pectin lyase, and cellulase. The endo-PG was detected earlier than the cellulase; it occurred concomitantly with germination and was easier liberated from the cells. The endo-PG also preceded pectin lyase in media simultaneously supplied with the respective inducers. However, it was stated that inducers for the production of one enzyme may be repressors for the production of another. Thus the cellulase inducers (carboxymethylcellulose (CMC)) and cellobiose repressed PG production.

The endo-PG had optima for activity at pH 4.0 and 5.0. The pectin lyase had considerable activity at pH 6.0, but the optima

were at pH 7.5 and 8.0 in the presence of $2 \cdot 10^{-3}$M $CaCl_2$.

Finally, attention is called to the essential results of Lai, Weinhold & Hancock (1968) stating that the endo-PG of *R. solani* increases the cell membrane permeability of mung bean (*Phaseolus aureus* Roxb.) hypocotyls to twice the rate of leakage of electrolytes and amino acids from the controls. Corresponding results were obtained by Hall & Wood (1973) with *R. praticola* and *E. carotovora* var. *atroseptica* with potato tissue and they correlated the efffect with the killing of plant cells (that is further discussed under the *Erwinia* species below).

Rhizopus species. *Rhizopus stolonifer* has a macerating (soft-rot) and toxic effect that is connected with the activity of PMG on sweet potatoes (Sherwood 1966, Spalding 1969). As oxydation was found to reduce the enzyme activity (compare the studies with apples, Cole 1956, Cole & Wood 1961a, b) extractions from soft-rotted sweet potatoes were made by squeezing between layers of cheese-cloth under an atmosphere of nitrogen and collecting the juice in a beaker with ascorbic acid. The enzyme was purified by acetone precipitation and dissolved in 0.1M phosphate buffer. This PMG had a strong macerating effect on sweet potatoes, turnip and cucumber, much less on potatoes, and very little on beets. The toxic effect measured as a decrease in respiration followed the same pattern (Spalding 1969).

Maceration was completely prevented by adding $Mg(NO_3)_2$ or $Ca(NO_3)_2$ in 0.1M concentrations, or KNO_3 in a 0.5M concentration. The inhibition appeared to be the result of the anions rather than of the cations, and in this context NO_3^- was more effective than Cl^- and SO_4^{--} (Spalding 1969). Basham (1974) purified an endo-PG from *R. stolonifer*, but as he apparently only studied its effect on Na-polypectate it is not clear if it is an endo-PG or an endo-PMG.

According to Brown & Wood (1953), Harter & Weimer (1923) were probably the first to demonstrate an adaptive production of 'pectinase' (PG), but these authors say that similar phenomena were found for other enzymes. The studies concerned *Rhizopus tritici*, which produces a powerful enzyme that dissolves the middle lamellae of raw sweet potato and carrot disks. The enzyme was only produced on vegetable media and not in artificial media unless pectin (obtained from carrot tissue) was added as carbon

source. The authors also tested other *Rhizopus* species and found that the most virulent species did not necessarily produce the greatest amounts of 'pectinase' under cultural conditions. In this connection it is mentioned that *R. stolonifer* caused soft-rot on sweet potatoes during storage, but only small amounts of enzyme in culture. Under the same conditions *R. tritici* also caused soft-rot, but it produced great amounts of enzyme in culture (Harter & Weimer 1923).

Zaitlin & Coltrin (1964) purified a commercial pectinase and an enzyme from a culture of *R. tritici* in order to study their effect on the intercellular cement of tobacco-leaf tissue. Both enzyme preparations exhibited PG activity with pectate as the preferred substrate, and the commercial pectinase was more active than that from *R. tritici*. In experiments with cell separation the former liberated cells (as % total chlorophyll) almost linearly with time during 1.5 hours of shaking. The latter showed a lag time of about half an hour after which the cell separation described a sigmoid curve reaching roughly the same level as the commercial enzyme in 3 hours. The lag time for the *R. tritici* enzyme was overcome by adding 10^{-3}M EDTA to the reaction mixture. Under these conditions, the activity of this enzyme equals that of the commercial pectinase. This result was compared to the fact that occasionally the commercial pectinase liberated only very few cells from greenhouse-grown leaves, and that also in this case the inhibitory effect could be removed by adding 10^{-3}M EDTA. This led to the suggestion that cations were involved, and a test with potassium, sodium, magnesium, and calcium salts, using maleate buffer instead of phosphate buffer in order to avoid precipitation of some of the salts, revealed that Ca^{++}-ions but not Mg^{++}-ions had a strong inhibitory effect on cell separation by the enzymes. Thus the inhibition might be caused by the presence of calcium. It has been stated that *R. tritici* produces one exo-PG and two endo-PG's (Rombouts & Pilnik 1972).

Sclerotinia species. This section should be compared with those dealing with *Sclerotinia sclerotiorum* and *Botrytis cinerea* (cf. paragraphs 6.1 and 6.2), both of which belong to this genus.

Sclerotinia fructigena and *S. laxa* were compared with *B. cinerea* by Cole (1956). When apples were attacked by *B. cinerea*

and *S. laxa*, the loss of insoluble and soluble pectin was greater than when they were attacked by *S. fructigena*. There was only little pectolytic activity in extracts from diseased apples, and culture filtrates from *S. laxa* showed only a weak macerating and viscosity-reducing effect, whereas these effects were pronounced for the other two fungi. The PE activity was considerable in all three fungi but highest in *B. cinerea*. The results (Cole 1956) show good accordance between the amount of mycelium and the PE activity, both increasing strongly with increasing concentration of glucose. In contrast, maceration and viscosity reduction were not much affected by glucose. Up until a pectin concentration of 1.5%, the effect was not much different from that of glucose as a carbon source, but at higher pectin concentrations the increase in PE activity slows down. At pectin concentrations higher than 1%, the macerating and viscosity-reducing activity is strongly reduced. The results with the mixture of glucose and pectin - as in many plants and storage organs - seem important. Mycelium production was greatest with 0.25% pectin and 0.75% glucose, and least with 1% pectin or 1% glucose. Maceration increased with pectin concentration until 0.75% and 0.25% glucose, and the PE activity increased in the same way. The viscosity loss per unit of time increased strongly with increasing pectin content. With apple pectin and Na-pectate the optimum pH was about 5 and with Na-polypectate about 6, strongly increasing from pH 5 and decreasing to pH 7 (Cole 1956). Cole & Wood's (1961a, b) findings are discussed under *B. cinerea* above.

In the course of studies with *S. fructigena* and *S. laxa*, Byrde & Fielding (1962) were the first to use dextran filtration on Sephadex G 75 for purification of pectolytic enzymes, but they still used Brown's (1915) old method to assay the activity. Using the new method the authors separated endo-PG activity from a thermolabile macerating factor at pH 4.7 using *S. fructigena* as test culture. Later, two isoenzymes of pectin lyase were separated and identified. Their activity was correlated with the maceration. One had optimum pH at 8.3 and was not activated by potato extract, the other had optimum at pH 7.3 and was activated by potato extract or Na-polypectate, while divalent cations had a negligible effect. In apple tissues rotted by *S. fructigena*, however, only small amounts of the pectin lyases were found; hence the authors conclude that these lyases

apparently have little and very localized significance in rotting the apple fruits (Byrde & Fielding 1968).

Byrde, Fielding & Archer (1973) review several papers on the pectolytic enzymes of *S. fructigena*. Based on their own experiments they state that the marked increase in PE in diseased apples was due to the pathogen and not to the host, and that dialyzed culture filtrate of the fungus grown on a polypectate medium rapidly induced cell leakage in apple tissue. By isoelectric focusing of healthy and infected apple tissue and of culture filtrates of *S. fructigena*, it was found that host PE had 3 components and that fungus PE had 2 components different from those of the host. There was no PG in the healthy tissue. The PG from the fungus had 2 components in both diseased tissue and in culture filtrates but at least one of them was different under the two conditions. *S. fructigena* did not produce lyase.

S. fructigena caused increased permeability in the rot edge as the electrolyte leakage from apple fruit tissue was twice as large within one mm of the edge than it was 1-2 mm away.

Howell (1975) made a correlation and regression analysis between the virulence of 119 isolates of *S. fructigena* - the majority of which were separated after mutagen treatment of conidia - and their in vitro production of three extra cellular cell-wall-degrading enzymes. They were α-L-arabinofuranosidase (AF), PG and PE. Analysed on untransformed data, only the interdependency of PG-AF, PG-PE, and AF in vivo growth was significantly correlated. When the interdependence was eliminated, there was only correlation between the virulence and AF. Unfortunately, the pectin lyase was not included in the analyses.

Held (1955) compared a normal strain of *S. trifoliorum* with a variant that on culture had lost its ability to wilt clover plants. Though Na-polypectate increases the secretion of protopectinase (macerating enzyme) from both strains, the non-pathogenic strain secreted more than the normal strain on artificial media. In the non-pathogenic strain, the secretion increased with the concentration of Na-polypectate while the normal strain secreted more at the lower concentrations. The normal strain produced a heat-stable toxic principle that rapidly wilted 'Ladino' clover leaves. The non-pathogenic strain did not.

Sclerotium rolfsii produces PG, PE and oxalic acid both in liquid artificial media and in bean hypocotyls during the pathogenesis (Bateman & Beer 1965). CMC was the best substrate for oxalate production. Pectin and glucose was less favourable. PG and PE were induced by CMC and pectin. The PG was unable to hydrolyze Na-pectate, but in the presence of oxalic acid ions it developed uninhibited. During the attack the pH dropped from 5.8-6.0 to pH 4.0, which is about the optimum for the PG. Bateman & Beer (1965) further indicated that the oxalate combines with the calcium in the pectate of the middle lamellae of the host cell walls. On this basis, a synergistic effect of PG and oxalic acid was supposed to be an important factor in the rapid destruction of the plant tissue. *S. rolfsii* did not produce lyase (Bateman & Beer 1965, Sherwood 1966).

The oxalic acid alone, or in combination with PG, caused the symptoms characteristic of the attacks of beans by *S. rolfsii*. At the same time the content of Ca^{++}-ions in the bean hypocotyls was inversely proportional to the susceptibility. These facts together underline the importance of Ca^{++}-ions for the resistance. Bateman (1972) separated the PG of *S. rolfsii* into two fractions, one with exo-activity and the other with both exo- and endo-activity. They both had an optimum pH of about 4, and were more active on polygalacturonic acid than on pectin. There were indications that both enzymes were active in bean hypocotyls as well as in culture. Crude preparations released galacturonic acid as well as oligomers in a 20-minute reaction time, while after 5 hours only galacturonic acid could be detected. Other experiments showed a 50% viscosity reduction with less than 1% hydrolysis and without any indication of the presence of lyases.

Thielaviopsis basicola produced in vivo and in vitro a calcium-stimulated hydrolase that was more reactive on pectin than on Na-polypectate at pH 4.5. This is the first observation of an obviously rarely occurring calcium-stimulated PG. Another case is reviewed below under *V. albo-atrum* (Mussell 1973). The fungus also produces a PG that is more reactive on Na-polypectate than on pectin at pH 6.0, and a calcium-stimulated lyase reactive on both pectin and Na-polypectate at pH 8 (Lumsden & Bateman 1966).

Verticillium albo-atrum. Early on, Bewley (1922) found that *V. albo-atrum* formed a substance capable of inducing wilting in tomato cuttings. The substance was thermolabile and precipitable with 50% ethanol. Later, Scheffer et al. (1956) found that culture filtrates from wheat bran cultures of *V. albo-atrum* caused wilting and vascular browning of tomato cuttings. After heating, the culture filtrate gave wilting but no vascular browning. Deese & Stahmann (1963) found a low PE and i high PG activity of *V. albo-atrum* growth on wheat bran. Grown on tomato plants, the enzyme production depends on the cultural variety (cv) and on the strain of *V. albo-atrum*. PE was produced in the susceptible ('Bonny Best') but not in the resistant ('Loran Blood') variety. PG was produced in large amounts from each of two strains on the susceptible, in moderate amounts from one of the strains on the resistant, and no PG was produced from the strains on the highly resistant variety ('VR Moscow'). The difference in production of pectolytic enzymes by *V. albo-atrum* on the tomato varieties was correlated with the oxidizing property and the quinone production. The susceptible variety gave no quinone reaction, the highly resistant gave a very strong reaction, while the resistant variety gave a reaction between these two. The inhibitory effect of the quinone is confirmed by Patil & Dimond (1967), who showed that the effect occurred when the quinone was produced in situ simultaneously to the activity of the *V. albo-atrum* PG.

The PG from the culture filtrate was found to be of the exo-type. The macerating activity was highest at pH 7. It was suggested that plant PE was implicated. This has optimum at pH 7 rather than at pH 5, which is the optimum for the fungal PE. The PG principally degraded the demethylated polygalacturonide chain of the pectin.

Talboys & Busch (1970) compared 42 *Verticillium* isolates distributed between 5 species, including *V. albo-atrum*. All isolates and species produced PE, PG and pectin lyase, but the amounts varied greatly within and between the species. PG and pectin lyase were constitutive, but their production was enhanced when pectin was present during growth. PE was produced on a pectin-glucose-salts medium but not on a potato-extract-pectin medium. Some correlation was observed between PE production and virulence. Seen as a whole, there was no close correlation between the activity of these pectolytic enzymes and the

virulence. However, in cases with PG and PE the latter may increase the activity of the former on highly methylated pectin.

Culture filtrates from growth on synthetic media containing $CaCO_3$ used for viscosity changes at pH 5 gave the closest similarity to the virulence. All three enzymes were present in these filtrates. Perhaps this is an interaction between the demethylation by PE and the formation of Ca-pectate and with the fact that the Ca-pectate forms a cell wall constituent. Further, Talboys & Busch (1970) stated that the pectin lyase activity increased in the following way in the presence of small concentrations of $CaCl_2$:

M $CaCl_2$	0	10^{-4}	10^{-3}	10^{-2}	10^{-1}
pectin lyase activity	100	110	125	147	128

The present author's results concerning lyase from *B. cinerea* are in close agreement with this.

Mussell & Green (1970) agree with these findings by concluding that PG production is correlated with the onset and development of disease symptoms caused by *V. albo-atrum* and *F. oxysporum* f.sp. *lycopersici* (see above) in susceptible tomato and cotton plants (*Gossypium hirsutum* L.). The authors could recover the fungi from infected resistant plants, but PG activity and disease symptoms were absent or much lower than in susceptible plants.

Wiese, DeVay & Ravenscroft (1970) tested 28 strains of *V. albo-atrum* and one of *V. nigrescens* for virulence and endo-PG production in vitro and found the two parameters inversely correlated. The results of Keen & Erwin (1971) are in agreement with this as they found no relationship between the endo-PG produced in culture or extracted from diseased plants. Non-purified preparations caused wilting symptoms, whereas purified endo-PG did not. Nevertheless the authors noted that two of the isolates exhibited a direct relationship as suggested by Mussell & Green (1970).

Keen & Erwin (1971) found the endo-PG of *V. albo-atrum* inducible, while Mussell & Green (1970) found it to be a constitutive enzyme, as also Mussell & Strouse (1972), who stated that the fungus produced copious amounts of endo-PG on glucose as sole source of carbon. The question of whether endo-PG is inducible or constitutive may, however, be too complex to be based only on the source of carbon, as stated by Cooper & Wood (1975), who found the inducer repressive when allowed to accumulate in the medium.

Mussell & Strouse (1972) separated two PG's from highly purified enzyme preparations from cultures of severely defoliating pathotypes of *V. albo-atrum*. Both enzymes had a pH optimum at 5-6, the lower for the activity on pectin, and they both exhibited higher activity on Na-polypectate than on pectin. One PG was of the endo-type and this had a relative macerating activity 20 times greater than the other PG that was of the exo-type. A separation of the reaction products by paper chromatography showed that the exo-PG liberated considerable amounts of galacturonic acid already after 1 hour's incubation, and that it was the only product after 24 hour's incubation. The endo-PG liberated several intermediates during the reaction time and only after 24 hours' incubation was galacturonic acid detected.

Finally, the authors mention that they have evidence leading them to believe that endo-PG function is a pathogenic determinant in the *Verticillium* wilt of cotton, because the endo-PG, but not the exo-PG, is phytotoxic to cotton leaves.

Heale & Gupta (1972) came to another conclusion as they could only detect the endo-PG in lucerne plants (*Medicago sativa* L.) in advanced attacks by *V. albo-atrum*. Instead, they found high amounts of exo-pectin lyases (4 components) as soon as it was possible to score any wilting symptoms. Small amounts of the exo-pectin lyase could be detected in root and shoots of healthy plants. Exo-pectin lyase, PE, and endo-PG were all constitutive, but their production enhanced in the presence of pectin or polypectate. An unidentified protein was further isolated from wilting lucerne. This protein markedly increased the viscosity of pectin and thus possibly contributed to the blocking of the vascular flow.

Mussell (1973) furthered his studies with the purified endo- and exo-PG and demonstrated that the symptoms caused by the

infection with *V. albo-atrum* were identical with those generated by the endo-PG. In this connection, he detected endo-PG-induced intramural hydrogen peroxide accumulation. Direct application of this compound to the cotton leaf tissue resulted in a rapid necrosis, but not of the intervenial type characteristic of *Verticillium* wilt. The apparent importance of hydrogen peroxide was confirmed by pretreatment of the leaves with catalase, which completely inhibited the activity of endo-PG. Other oxidases caused leaf damage when applied after pretreatment with their specific substrates. Mussell (1973) also made the important observation that divalent cations strongly enhance the activity of the endo-PG. While KNO_3 and $NaNO_3$ gave a leaf damage index of 1.2 like water, $Ca(NO_3)_2$, $Mn(NO_3)_2$ and $Mg(NO_3)_2$ gave indexes of 2.5, 3.2 and 4.8, respectively. Only once before has a calcium-stimulated PG been observed. It was produced by *Thielaviopsis basicola* (Lumsden & Bateman 1966), see the discussion above. Mussell (1973) drew attention to the correlation between the constitutively produced endo-PG and the pathotype, but not directly with the virulence of each isolate within the pathotype. This is in agreement with Deese & Stahmann (1963) and Talboys & Busch (1970).

Basham (1974) compared purified endo- and exo-PG from *V. albo-atrum*. They had optimum pH at 5.3 and 6.0, respectively. The former caused maceration and cell death in potato tissues while the latter caused little maceration and no cell death.

The studies on the endo-PG and endo-pectin lyase produced by *V. albo-atrum* are discussed above together with *Fusarium oxysporum f.sp. lycopersici*. Here it should only be mentioned that they were both found to be adaptively induced, but repressed by an accumulated inducer (Cooper & Wood 1975).

By use of mutants obtained as a result of UV-irradiation, Puhalla & Howell (1975) overcame some of the problems discussed in connection with the contradictory results obtained by others. The authors assayed more than 60,000 viable irradiated conidia of *V. dahliae* (they treated *V. dahliae* as synonymous with *V. albo-atrum*, which it is not) and found 0.01 to 0.04% of the conidia to lack the ability to produce detectable amounts of endo-PG. From each of three parent isolates, they further tested two mutants which, although without endo-PG, gave the same disease index as the parents. All but one of the mutants also had

the same cellulase activity as the parents. Thus, regardless of the importance of other parameters, these results show that endo-PG is not necessary for the virulence of *V. dahliae*. Concerning the change in enzyme production, Friedman & Ceponis (1959) obtained similar results with *Pseudomonas marginalis* (see below), but with much higher frequency, and Albersheim, Jones & English (1969) reported on other, analogous cases.

9.1.2. Bacteria

Aeromonas liquefaciens (rejected as unrecognisable in Bergey's Manual 1974) which, according to Hsu & Vaughn (1969), causes softening of plant material, produces pectate lyase. Production of the enzyme was strongly stimulated in media giving restricted growth, e.g. caused by limited nutrient supply or divalent cations in continuous culture, or with polygalacturonic acid as the sole source of carbon. This is obviously due to catabolite regression of the enzyme formation (cf. e.g. Cooper & Wood 1975). The authors stated the enzyme to be constitutive and found a mutant producing the pectate lyase independent of the amount of nutrient supply.

The effect of increasing concentrations of EDTA on the production of pectate lyase in a medium with 0.2 g each of $CaCl_2$ and $MgSO_4,7H_2O$ was compared. Enzyme activity was very slight during the incubation time without EDTA and variable at concentrations below 0.02%. Above this value the activity increased with concentration in the medium up to 0.08% EDTA, which may have removed almost all the divalent cations from the medium and thereby made the polygalacturonic acid, used as sole carbon source, more easily accessible.

The enzyme activity was assayed in a reaction mixture with 10^{-3}M $CaCl_2$ and 0.033M tris-HCl buffer at pH 8.

Bacillus and *Clostridium species*. *Bacillus* species are saprophytes under normal conditions but *B. megaterium* and *B. subtilis* may contribute to the maceration of potato tuber tissue if its turgescence is raised above the normal level (Fernando 1937, Fernando & Stevenson 1952). However, they may or may not contribute to the retting process of flax (Allen 1946, Smith 1958a, b, Rosenberg 1965). Also *B. polymyxa* may cause soft-rot

in potatoes, and it may be active in spoiling canned fruits and vegetables. Probably more important is its participation in the wet retting of plant materials under aerobic as well as anaerobic conditions. *B. polymyxa* produces a PE and a pectolytic enzyme identified as an endo-pectate lyase more active on pectic acid than on pectin (Allen 1946, Hellinger 1954, Smith 1958a, b, Nagel & Vaughn 1961a, b, 1962, Starr & Moran 1961, 1962). Both enzymes were inducible with pectin as the best inducer followed by pectic acid and galacturonic acid (Nagel & Vaughn 1961a, b, 1962). Rombouts (1972) noted that *B. polymyxa* produces endo-pectate lyase and PE, whereas *B. subtilis* produces both endo-pectate lyase and PG. The behaviour of other *Bacillus* species is reviewed by Rombouts & Pilnik (1972), who noted that the calcium required for the lyase activity could be replaced by strontium or cobalt.

The lyase had optimum about pH 9 and an absolute requirement for Ca^{++} that increased the activity with increasing concentration up to about 10^{-3}M $CaCl_2$, while higher concentrations proved inhibitory. The lyase activity could be completely stopped by EDTA (Nagel & Vaughn 1961a, b, 1962, Starr & Moran 1962). Fractionation on CMC followed by treatment on a DEAE-cellulose column gave four different fractions that appeared to degrade pectic acid in the same manner. Both the crude enzyme and the fractions degraded trigalacturonic acid to an unsaturated dimer and galacturonic acid. The tetramer gave monomer and unsaturated trimer or normal and unsaturated dimer. The digalacturonic acid was not degraded by the lyase, but *B. polymyxa* produced a digalacturonidase that splits both the normal and the unsaturated dimer. This enzyme was exudated during the logarithmic growth phase and by lysis of the cells.

The wet retting is mainly an anaerobic process in which *Clostridium* species are primarily responsible (Rosemberg 1965), and hence their pectolytic activity has been studied in several cases.

After microscopic examination, van Tieghem (1877, quoted by Störmer 1903) claimed that his *Bacillus amylobacter* was active in the maceration process, but he did not work with pure cultures such as necessary for unambiguous results (Störmer 1903). However, by using authentic cultures of *C. butyricum*, Hellinger (1954) found the bacterium completely inactive on Na-

pectate and in the retting process. Neither could Hellinger find any pectolytic or retting activity from authentic cultures of *C. pectinivorum* (which she found identical with *C. tertium*), in contrast to Störmer's (1903) statement concerning his pure culture of this bacterium.

Most active in retting under anaerobic conditions and in the degradation of pectic substances were *C. aurantibutyricum* and *C. felsineum*, while the pectolytic *B. polymyxa* was stated to be moderately active in the retting process, and more under aerobic than under anaerobic conditions. For comparison, Hellinger included the plant pathogen *Erwinia carotovora var. carotovora* in her experiments and found it to exhibit an activity like that of *B. polymyxa* under aerobic conditions (Allen 1946, Hellinger 1954). *B. polymyxa* produces at least a calcium-dependent endo-pectate lyase (Starr & Moran 1962).

Tani (1967) mentions that the 'macerating enzyme' of *C. felsineum* resembles the enzyme of the kaki fruit. This enzyme is discussed above under *Colletotrichum gloeosporioides*. However, it also produces a PG that cannot macerate plant tissue (Kaji 1958, in Bateman & Basham 1976).

Apparently, inoculation with *C. felsineum* is used directly for shortening the process of wet retting in Russia (cf. e.g., Avrova 1975).

Several other bacteria may contribute to the process of wet retting (see, e.g., Rosemberg 1965). In dew retting, different fungi are mainly responsible, e.g., species of *Aspergillus*, *Cladosporium*, *Penicillium*, and *Rhizopus* (Allen 1946).

Finally, it should be mentioned that *Clostridium multifermentans* produces an exo-pectate lyase adaptively with optimum activity at pH 8.5 in the presence of 0.5×10^{-3}M $CaCl_2$. With increasing calcium concentrations, the reaction rate was progressively retarded, or stopped on a lower level of hydrolysis, analogous with the activity of PE in the present author's experiments. The requirement for Ca^{++}-ions was not specific as it could be replaced by the following cations listed in order of the reduced stimulatory effect of 10^{-3}M concentrations: Ca^{++}, Sr^{++}, Mn^{++}, Mg^{++}, Ba^{++}. Zn^{++} was without any effect. The addition of EDTA completely stopped the activity.

The crude enzyme accompanied by PE degraded highly esterified pectin, but in the purified state there was no activity on

pectin. The exo-pectate lyase is much more resistant to pH and temperature than is the PE, and these facts are used in the purification procedure.

This exo-pectate lyase splits the molecular chain from the reducing end and leaves principally unsaturated digalacturonic acid in the reaction mixture (MacMillan, Phaff & Vaughn 1964, MacMillan & Vaughn 1964, MacMillan & Phaff 1966, Miller & MacMillan 1970).

Erwinia species. Much work has been devoted to studying the pectolytic activity of the soft-rot bacterium *Erwinia carotovora* in sensu lato. Some of the work does not specify the strains, in others the strains are specified as *E. carotovora var. carotovora* and *E. carotovora var. atroceptica* (cf. Buchanan & Gibbon: Bergey's Manual of Determinative Bacteriology 8th ed. 1974 for the systematics of these bacteria) or their synonyms.

Buston & Kirkpatrick (1931) observed that *E. carotovora* macerated the phloem parenchyma (p.p.) and the stele of carrots at a different rate. They did not, however, find any differences in the character of the pectic substances, only that the stele contained about half the amount of those in the p.p., and that the difference mainly resulted from a higher amount of protopectin (water-insoluble pectin) in the p.p. *E. carotovora* degraded pectin isolated from the stele or the p.p. of carrots equally well.

In comparison with *B. cinerea* and *P. debaryanum*, it was found that *E. carotovora* gave the same pH response as did *P. debaryanum*; i.e. an optimum at pH 8 decreasing to a minimum at about pH 5.5, where *B. cinerea* has its optimum (Fernando 1937, Fernando & Stevenson 1952). Tribe (1955) agreed with this finding and added that the enzymes killed the cells of the macerated tissue, although he did not leave the possibility of a thermostable toxic compound out of account (compare the discussion of *B. cinerea*). The dead cells were detected by the excellent neutral red method (NR-index method) of Tribe (1955) described in paragraph 5.3.3.3. Tribe stated that the removal of calcium from the culture filtrates of *E. carotovora* inhibits maceration and the killing of cells.

In *E. carotovora var. carotovora* 'pectinase' is apparently a constitutive enzyme, unlike the case in many fungi (Brown & Wood 1953). This at least applies to the depolymerase found by Wood (1955). This 'pectinase' is of the type that causes a pronounced decrease in viscosity of pectic substances which is simultaneous with only a small reducing power of the reaction products, and it is more active on pectate than on pectin. Hence it is an endo-pectate enzyme, but at the time of these studies it could not, of course, be determined whether it was a hydrolase (PG) or a trans-eliminase (lyase). In all probability it was the latter because of the optimum pH 9.0 and its dependence on Ca^{++}-ions. The enzyme exhibited no activity on dialysis, but the addition of 0.2-4 × 10^{-3}M $CaCl_2$ gave increased activity. The latter concentration gave an activity almost equal to the original undialyzed enzyme. Comparing the activity of the enzyme and the enzyme plus 2 × 10^{-3}M $CaCl_2$ at pH values from 7.0 to 9.0, showed that calcium gave a 9- to 15-fold increase with pH up to 8.5. Ion-exchange resin greatly inhibited enzyme activity.

In addition to the above-mentioned enzyme, Tribe (1955) found that the bacterium also produced a protopectinase (macerating enzyme), which, by using Brown's (1915) method, increased the macerating activity of potato tissue with increasing pH at least up to 9.6. Though obviously not dependent on calcium, this enzyme was greatly stimulated by the addition of 5 × 10^{-3}M $CaCl_2$ to the dialyzed enzyme.

In the USA the terms "watery soft-rot" and "slimy soft-rot" are used to describe the types of rot caused by *Sclerotinia sclerotiorum* and *E. carotovora*, respectively. This difference is thought to be an expression of the activity of the pectolytic enzymes of the two organisms. Echandi & Walker (1957) revealed that the former produced PE and PG with an optimum pH of 4-5, while the latter was stated to produce a pectin depolymerase that cut the molecular chain into smaller pieces (see above) with an optimum pH of 9 and no PE or PG (cf. the discussion of *S. sclerotiorum* above and the results of the present author). The authors also observed that *S. sclerotiorum* penetrated the cells directly, which *E. carotovora* did not.

Among the large number of bacteria examined for pectolytic activity by Smith (1958a, b) were two strains of *E. carotovora var. carotovora* and five strains of *E. carotovora var. atrosep-*

tica that produced both PE and 'PG', while one out of five strains of *E. carotovora* did not produce PE. This is in agreement with some authors, but not with others, however, this inconsistency may possibly may explained by the fact that the adaptibility of the enzyme was not always taken into account (cf., e.g., Starr 1959, Starr & Chatterjee 1972, Bonnet & Venard 1975).

In comparisons between *Pythium debaryanum*, *Botrytis cinerea* and *E. carotovora* var. *carotovora* - discussed in more detail under *B. cinerea* - it was found that the enzyme of *E. carotovora* var. *carotovora* that degraded pectic substances at pH 8.3 was of the endo-type because it produced two intermediary compounds and no galacturonic acid. The rate of activity was higher for highly methoxylated pectin than for other pectic substances (Wood & Gupta 1958).

Starr & Moran (1961, 1962) found that both *B. polymyxa* and *E. carotovora* produced an endo-pectate lyase that was more active on pectate than on pectin. Its activity could be completely removed by adding EDTA. The activity was restored by adding $CaCl_2$ and it increased with concentrations up to about 10^{-3}M, while higher concentrations proved inhibitory.

Dean & Wood (1967) passed dialyzed culture fluid from growth of *E. carotovora* var. *carotovora* through CMC and DEAE cellulose columns and tested the fractions for pectolytic and macerating activity. It was thus revealed that the bacterium produced both PG and pectate lyase, and that the macerating activity was closely correlated with the latter. This fact was further established by the results of Mount, Bateman & Basham (1970), who found that the endo-pectate lyase of *E. carotovora* caused electrolyte loss, tissue maceration, and cell death of potato tissue. Further, their results led to the suggestion that a substrate for the enzyme resides in the plant cell membrane or within the protoplast. These experiments were performed in tris-HCl buffer at pH 8 in the presence of 10^{-4}M $CaCl_2$, in which the electrolyte loss was measured by leaching out accumulated ^{86}Rb from the cells, and cell death by the neutral red method of Tribe (1955). A comparison of the results shows that the permeability changes and most of the isotope release precede cell death (Mount, Bateman & Basham 1970, Wheeler 1975).

Based on experiments using highly purified endo-pectate lyase from *E. carotovora* with 10^{-3} - 8×10^{-3}M $CaCl_2$ at pH 9,

Stephens & Wood (1974, 1975) stated that the enzyme leads to the release of K^+-ions and several enzymes from tissue at the same time as it causes maceration and death of the protoplasts. The crude enzyme caused complete maceration and cell death in less than two hours in normal tissue of potato, carrot and cucumber. In plasmolyzed tissue the process took about six hours in potatoes and 24 hours in carrots, while after six hours there was no cell death in cucumber. The cell death may probably be explained as a rupture of the protoplasts under turgor pressure due to an alteration of the cell wall structure brought about by the enzyme, hence the much reduced effect on plasmolyzed tissues. In agreement with the results with cucumber, Tseng & Mount (1974) found that purified endo-pectate lyase from *E. carotovora* did not cause lysis or loss of neutral red from isolated cucumber protoplasts.

E. carotovora var. carotovora also produces an exo-pectate lyase that is hardly active on 70% esterified citrus pectin. The enzyme is scarcely affected by calcium (Rombouts 1972).

Finally, it should be mentioned that *E. carotovora* produces an oligogalacturonide lyase that cleaves both digalacturonic acid and unsaturated digalacturonic acid. The activity of this enzyme is inversely proportional with the length of the chain pieces of the molecule. This lyase has optimum pH at 7.2 and is independent of calcium ions (cf. Starr & Chatterjee 1972). These facts may perhaps explain some of the confusion in the literature about the exo- and endo-types of enzymes.

The synergistic effect of the pectolytic enzymes from *E. carotovora var. carotovora* and *Phytophthora cryptogea* (El-Goorani, Abo-El-Dahab & Khoshnow 1976) is discussed above under the fungus.

The cause of resistance to attacks by *E. carotovora var. atroseptica* was studied by Stapp & Hartwich (1957), who stated that the formation of a cork barrier in wounds of *Solanum* species could not be the only decisive factor in the resistance. These authors raised the question of whether the proportion of Ca to pectin might be of importance for the resistance, but their experiments did not exhibit a clear relation between the resistance and a high Ca/pectin quotient. This lack of a relation could probably be because the effect of calcium varies, depending on which physiological system it is coupled up with in the differ-

ent cases. Thus the authors found that Ca^{++} increased the resistance and Na^{+} increased the susceptibility, but they could not determine whether pectolytic enzymes were active in this connection.

E. carotovora var. atroseptica spreads intercellularly and occurs primarily in the intercellular spaces of the calcium oxalate cells of the phelloderm and in the vascular tissue, but not in the vascular parenchyma, which was suberized even in healthy potatoes. Some damage to storage parenchyma could occur up to 500 µm from the site of infection (Fox, Manners & Myers 1972). In comparison with healthy tissue it was revealed that infected storage parenchyma lacked pectic substances in the region of the middle lamella and that the intercellular spaces were much enlarged. The cell walls of the rotted tissue swelled with constrictions in the plasmodesmata, but it was not ascertained whether or not there was a change in the cell surface area. The authors stated that suberin formation in vascular parenchyma constitutes a barrier to infection. Further, they asserted that any method for promoting, stimulating or simulating the intercellular barrier of suberin by genetical or chemical means would obviously impede bacterial soft-rot (Fox, Manners & Myers 1972). Confer the wound healing process in connection with the attack by *E. carotovora var. atroseptica* (Smith & Smart 1959). Further, the suberization of potatoes may give some protection against storage rot after irradiation (Skou 1978 XI).

A comparison of the rapidly developing soft-rot caused by *E. carotovora var. atroseptica* with the slowly developing rot caused by *Rhizoctonia praticola* clearly showed the differences between the pectolytic enzymes of the two organisms. The former produced an endo-pectate lyase with optimum pH above 9.0 and was activated by Ca^{++}-ions (10^{-3}M). The latter produced an endo-PG with optimum pH at 4.0-5.0 and was inhibited by Ca^{++}-ions. The enzymes were free of other pectolytic enzymes and both caused maceration, increased permeability of the potato tuber cells, and cell death - which effects are interrelated (Wood 1972, Hall & Wood 1970, 1973) (cf. Figure 53). In this connection it is worth noting that the electrolyte leakage caused by the lyase of *E. carotovora var. atroseptica* was greatest with calcium present in test solution. As soon as the potato tuber cells came into contact with the enzymes, there was a rapid in-

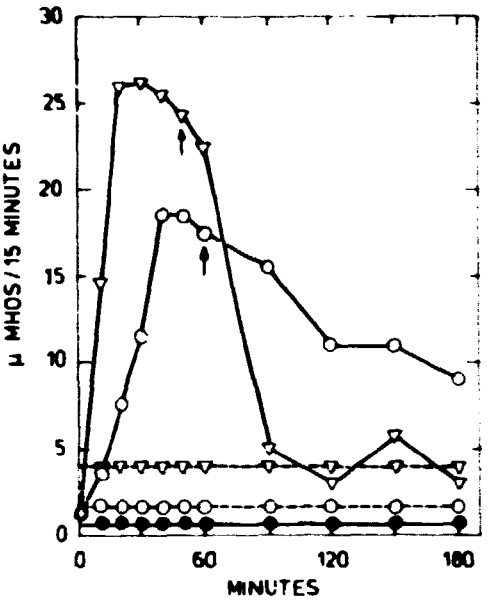

Figure 53. Leaching out of electrolytes from potato tissue as a measure of permeability of potato tuber cells.---: boiled enzyme solutions; ●: in water; ○ : enzyme from *Erwinia carotovora var. atroseptica*; ▽ : enzyme from *Rhizoctonia praticola*. The arrows give the time for loss of coherence of the cells. (Drawn after Hall & Wood 1973, Table 1).

crease in permeability measured as the leaching out of electrolytes per unit of time. After 30-40 minutes the rate of leaching reached a maximum, and shortly after the cells lost their coherence. This increase in permeability should be compared to that caused by *Botrytis cinerea* and *Sclerotinia sclerotiorum* as discussed above (cf. Thatcher 1939, 1942).

On this basis, the authors (Hall & Wood 1970, 1973) suggested that the killing of cells by pectolytic enzymes may primarily be a physical effect by osmosis in which the plasmalemma is ruptured at one or a number of points following the degradation of substrates within the middle lamellae or cell wall. The rupture comes about either by hydrolysis (PG) or by trans-elimination (lyases). The same suggestion applies to many plant pathogens (Wood 1972).

It should, however, not be disregarded that the presence of phosphatidases (e.g., *P. glaucum*) may play a role in connection with cell death (Wood 1972, Hall & Wood 1973).

The pectolytic activity of *E. chrysanthemi*, as studied by Hellmers (1958), was verified by Gehring (1961/62) when he described the great differences in activity between the many isolates tested regarding their effect on potato tissue. The results were in agreement with the activity exhibited on pectate gel plates.

Rombouts (1972) tested many species and strains of bacteria for the type of pectolytic activity and found that *E. carotovora* varies as shown in Table 33.

Table 33. Differences in pectolytic activity of strains of *Erwinia carotovora*. From Rombouts 1972.

Bacterial strain	PE	PG	Pectate lyase		Pectin lyase
			exo	endo	
E. carotovora	+	+	-	+	
E. carotovora G 117	-	+	+	+	-
E. carotovora var. *atroseptica* (*E. atroseptica* SR1)	+	+	-	+	-
E. carotovora var. *carotovora* (*E. aroideae* 140 V)	+	+	-	+	+

Just as *E. carotovora* and its varieties produce an endo-pectate lyase with optimal activity at an alkaline pH, so does *E. chrysanthemi*, and at least some of its isolates produce more than one. Thus, Garibaldi & Bateman (1971) had an isolate with two and another with four different pectate lyases, which attacked the pectic polymers in different fashions. The enzymes were separated from each other by $(NH_4)_2SO_4$ fractionation, DEAE-cellulose column chromatography and isoelectric focusing. The isoelectric point of the enzymes differed from pH 4.6 to pH 9.4, while their optima for activity varied from pH 8.2 to

pH 9.8. They all exhibited requirements for calcium ions, and all but one of the highly purified lyases induced maceration, electrolyte loss and cell death of potato tissue. The enzyme with an isoelectric point at pH 4.6 did not macerate tissues from potato, carrot or cucumber, even though it readily degraded pectic acid from the plants as well as Na-polypectate, polygalacturonic acid, and pectin (N.F.). The occurrence of pectate lyase isoenzymes in strains of *E. chrysanthemi* was confirmed by Pupillo, Mazzucchi & Pierini (1976), who on the basis of the isoelectric points distinguished between "alkaline" (pH 9.7 to 9.0), "central" (pH 8.7-7.8) and "acid" (pH 4.7-5.0) isoenzymes. Up to four isoenzymes were detected in a strain in agreement with Garibaldi & Bateman (1971).

Here, an important statement by Beraha & Garber (1971) and by Beraha, Garber & Billeter (1974) should be mentioned that the avirulent strains of *E. carotovora* produced little PG, lyase and cellulase, in contrast to the activities in the parental virulent strain and revertant strains. This means that the enzymes (PG and lyase) are responsible for the virulence in this organism.

The enzyme components that exhibited endo-pectate lyase killed tissue at about the same rate as it was macerated, whereas the components that exhibited exo-pectate lyase activity tended to kill the tissue at a faster rate than it was macerated. However, there were differences between the tissues of different plants and the killing could be delayed by plasmolysis (Garibaldi & Bateman 1971). All these facts indicate a direct toxic effect on plant cell protoplasts or membranes in agreement with that found by Hall & Wood (1973).

Basham (1974) made a very pure preparation of endo-pectate lyase from *E. chrysanthemi* by adsorption on CM-Sephadex, DEAE-cellulose, CM-Sephadex column chromatography and isoelectric focusing which, by gel electrophoresis, showed a single protein band able to cause lytic degradation of Na-polypectate, and to cause maceration and cell death of potato and tobacco tissue while sugar beet tissue proved resistant. Basham (1974) characterized the injury of the cells by the rate of loss of ions and water from the cytoplasm, and found the rate of electrolyte loss was correlated with the rate at which the unsaturated uronides were brought into solution. In agreement with other authors,

Basham (1974) also found the cells to be protected against loss of ions at incipient plasmolysis in the presence of the enzyme, but not against degradation of the cell wall. These facts indicate damage to the plasmalemma. The results were further confirmed by Basham & Bateman (1975a), who found proportionality between the following parameters: Enzyme activity and cell death, maceration and cell death, cell wall degradation and electrolyte loss, loss of unsaturated uronides and electrolyte loss, and weight loss (by the blotting and weighing method, cf. Skou 1963 I) and electrolyte loss. Only endo-hydrolases and endo-lyases caused both maceration and cell death, whereas an exo-hydrolase only caused weak maceration and no cell death. The contact between plasmalemma and endo-pectate lyase of *E. chrysanthemi* did not result in membrane damage, and removal of the enzyme from cell-wall-degraded, plasmolyzed tissue caused no harm, but the following deplasmolysis led immediately to cell death. These facts support the hypothesis that injury to cells in plant tissue treated with pectolytic enzymes results from a loss in the ability of enzymatically degraded cell walls to support the plasmalemma of living cells (Basham & Bateman 1975b).

Bonnet & Venard (1975) found it possible to distinguish between *E. chrysanthemi* and the varieties of *E. carotovora* as the former produces PE and the latter does not. This is in agreement with some authors (cf. Husain & Kelman 1959) but not with others (cf. Smith 1958a, b, Starr 1959, Rombouts & Pilnik 1972, Starr & Chatterjee 1972).

Pseudomonas species. Smith's (1958a, b) survey shows the presence of pectin glucosidase (PG-type) in one out of three strains of *Pseudomonas marginalis*. The enzyme was stated to liberate mono- to penta-galacturonic acids. Friedman & Ceponis (1959) demonstrated the connection between soft-rot and the pectolytic activity from PE and depolymerase (endo-type degradation) of *P. marginalis*. After exposure to a germicidal UV-lamp, they separated ten mutants which were unable to cause soft-rot and failed to produce pectolytic enzymes. *P. marginalis* may produce PE, PG, and pectate lyase, but the amount strongly depends on the strain and on the carbon source used (Nasuno & Starr 1966). This shows how difficult it is to generalize about the pectolytic properties of a species.

P. solanacearum produces relatively much PE and PG (Winstead & Walker 1954) in comparison with *B. cinerea*, *Fusarium* spp. and other fungi. One out of five strains of *P. syringae* produced pectin glucosidase (PG-type) and several other *Pseudomonas* species exhibited no pectolytic activity at all (Smith 1958a, b).

Preiss & Ashwell (1963a, b) isolated a pectolytic enzyme from sonic disrupted cells of a *Pseudomonas* sp. that they termed polygalacturonase, although its activity resulted in the formation of unsaturated oligogalacturonides which were further degraded to the unsaturated monomer. However, the difficulties with the terminology are understandable as the authors also found that the same enzyme was responsible for the accumulation of the saturated galacturonic acid. The endo-products of the reaction were further metabolized to pyruvate and a triose.

Like other lyases, the enzyme had an optimum pH at 8.3-8.5 and an absolute requirement for calcium ions.

The effect of salts was a matter of detailed investigation as their presence clearly inhibited the enzymatic activity of the crude extract as well as of the purified enzyme.

Each of the following salt concentrations resulted in a 50% inhibition: 0.05M KCl, 0.07M NaCl, 0.02M K_2HPO_4, and 0.02M K_2SO_4. A concentration of 10^{-4}M $MnCl_2$ gave 90% inhibition, whereas 10^{-4}M $MgCl_2$ gave 40% stimulation which diminished to no effect on increasing concentrations. In the crude extract, 10^{-4}M $CaCl_2$ was without effect, and 10^{-3}M $CaCl_2$ caused 32% inhibition. On the other hand, the chelating agent, EDTA, also proved inhibitory as 3×10^{-5}M EDTA caused 16% inhibition that increased to 93% at 10^{-4}M, and dialysis of the enzyme against 10^{-2}M EDTA for two days followed by dialysis against water for a further two days diminished the activity to one third of the activity of the untreated enzyme. The activity was fully restored by 2×10^{-4}M $CaCl_2$. 10^{-4}M $CaCl_2$ caused a 2-fold stimulation of the activity of the purified enzyme. Higher concentrations proved inhibitory and above 10^{-3}M $CaCl_2$ the polygalacturonate precipitated as a gel (compare Table 17).

Other bacteria. As in the case of the phytopathogenic microorganisms, pectolytic activity is a common property of the saprophytes, which may therefore contribute to the degradation of

plant litter and other deposits of plant material. Some of these organisms are discussed under *Bacillus* and *Clostridium* above in connection with the retting of plant material.

Smith (1958a, b) tested many species and strains of saprophytic and plant pathogenic bacteria for pectolytic activity. Of the former group, 15 strains out of 56 produced γ-pectin-glycosidase (most active at pH 7.5), and probably a lyase, whereas the corresponding figures for the latter group were 22 strains out of 67 tested. PE was produced by a few plant pathogens but not by the saprophytic strains. Generally, there was a great variation in pectolytic activity within as well as between the species. Here it should only be mentioned (Smith 1958a, b) that 'PG' is produced by *Bacillus*, *Pseudomonas*, and *Xanthomonas* species, but not by *Agrobacterium* and *Corynebacterium*. In these investigations only species of *Erwinia* and *Xanthomonas* produced both 'PG' and PE.

Bacteria of the coli-aerogenes group may or may not produce pectolytic enzymes. Thus Taylor (1951, cited by Smith 1958a) found that 17 out of 19 soft-rot bacteria of *E. carotovora* type liquified pectate gel, whilst some 300 other coli aerogenes bacteria did not. On the other hand, strains of *Citrobacter* and *Klebsiella* produced 'PG', whereas *Escherichia coli* (Mig.) Cast. & Chal. proved negative (Smith 1958a, b).

Rombouts (1972) tested several bacteria for pectate lyase activity. Of these, *Athrobacter* 547 was studied in most detail. It produces pectate lyase with a pH optimum at about 7.0 and like other lyases it is calcium dependent (Figure 54). Calcium and magnesium greatly stimulated the reaction rate, whereas strontium and manganese were without effect, and barium was inhibitive compared to no addition of salt. EDTA completely stopped the lyase activity.

Rumen bacteria constitute a specialized group of saprophytes several of which are not only able to degrade pectic substances, but also to use them as the sole source of energy (Gradel & Dehority 1972, and others). This process facilitates metabolism of the plant material.

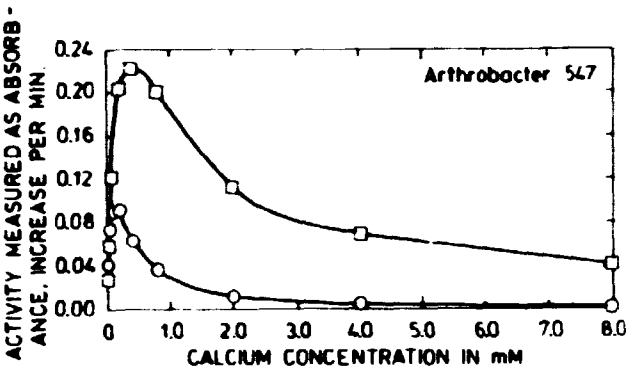

Figure 54. The effect of calcium ions on the activity of pectate lyase from *Arthrobacter* 547 in 0.25% (□) and in 0.025% pectate (○). (Redrawn after Rombouts 1972, Figure 32).

9.1.3. Higher Plants

Pectolytic enzymes seem widely distributed in the higher plants, and may, as mentioned in the discussions above, interact with the enzymes of the pathogens. Only a brief review will be given here in order to outline the similarities and differences between the pectolytic enzymes of hosts and pathogens.

The first discovery of an enzyme active on pectin was made by Frémy in 1840 with the detection of pectinesterase in carrots (Matus 1948).

Carrot tissues contain a polygalacturonase that slowly lowers the viscosity of a pectin solution as it degrades the molecular chain from the non-reducing end by liberating galacturonic acid and only hydrolyses 14% of the glycosidic bonds (Ozawa 1955, Ozawa & Okamoto 1955a, b). The enzyme was stated to be an exo-PG exhibiting higher activity on partially degraded pectic acid than on, e.g., pectic acid from citrus pectin, or from carrot roots, and with a low activity on that from peach flesh. In no case was the hydrolysis complete. The pH optimum of the enzyme lies in the range 4.7-5.6 (Hatanaka & Ozawa 1964). It should be the only enzyme in carrots attacking the molecular chain.

Cranberries (*Oxycoccus macrocarpon* (Ait.) Pursh.) contain an endo-PG that is active on pectin both at a high and at a low

level of methoxylation. The enzyme had a higher activity on citrus pectin than on cranberry pectin. Optimal activity at pH 5.0. Use of a phenol-binding agent was necessary in order to obtain a high enzyme activity. Adding up to 0.6M NaCl gave no effect.

The cranberries exhibited low PE activity compared to that of strawberries and tomatoes. Like the PE's from other plants, the enzyme had a pH optimum at 7.5, which is clearly different from the PE's of microbial origin with an optimum pH at about 5.0 (Arakji & Yang 1969).

Bell (1951/52) observed that cucumber fruit tissue became soft in salt-stock and found the cause in a PG isolated from the brine. Further studies revealed that the enzyme originated from the cucumber fruits. Extractions from various parts of the plants showed that leaves, petioles, stems, and unpollinated flowers were without PG activity. Green fruits of various sizes had a small activity, ripe fruits and seeds had moderate to high activity, and only the stamminate and the pollinated flowers had a very high activity. Bell (1951/52) also found a PE in the cucumbers, but he prevented its interference by incubating the extracts at pH 3.0 for 24 hours at 40°C, which destroyed the PE and left the PG little affected.

The coleoptiles of oats contain a PE that is strongly bound to the cell walls. Its properties are not principally different from those of orange fruit PE (MacDonnell, Jansen & Lineweaver 1945, Jansen, Jang & Bonner 1960a, b). In orange juice, PE was found bound to cell wall pieces from which it could be solubilized by 0.15M NaCl at pH 7.5, the process being promoted by the addition of soluble pectin. The free enzyme rapidly de-esterified pectin and had a strong affinity to de-esterified cell walls. Optimum pH was 7.5, but activity could also be measured at pH 4.5. When bound to the cell walls, however, it was inactive at this pH.

In this connection it is worth mentioning that Somers (1973) found that onion cell walls exhibit a high affinity for Ca^{++}-ions, and that the adsorption is enhanced by the action of PE. The calcium ions could be displaced by high concentrations of magnesium or potassium relative to that of calcium.

McColloch & Kertesz (1947) compared tomato PE (t-PE) with fungal PE (f-PE) (commercial Pectinol PM) and found decisive

differences, which were confirmed by Miller & McColloch (1959) and Bateman (1963a). These differences are summarized in Table 34 and may be taken as generally applicable to distinguishing the PE's of plant and microbial origin.

Table 34. Comparison between pectinesterase from tomato and from fungi.

	t-PE	f-PE
Optimum pH	about 7.0	5.0-5.5
Inactivation temperature	60°C or above	20-25°C
NaCl*	dependent for activity; necessary for extraction from plants	independent
Detergents	sensitive	resistant; 100 times the quantity is required to reach the same level of inhibition**
General enzyme inhibitors	relatively resistant	relatively sensitive
Affinity to plant cell walls	adsorbed or bound	not adsorbed or bound

* Possibly a salt effect. Lumsden (1976) also detected this effect using $CaCl_2$.

** The inhibitory effect on *R. solani* PE was 25% for 75 ppm and 72% for 150 ppm. The PG was more retarded; namely 57, 90 and 100% for 75, 150, and 500 ppm respectively (Grossman 1968).

After the action of PE, the demethyloxylated pectin will give a precipitation of Ca-pectate or Ca-pectinate, but if PG is present during the PE action there will be no precipitate because of the simultaneous hydrolysis of the polygalacturonic acid. Probably PE enhances the activity of these types of PG that act more rapidly on pectate than on pectin. On the other

hand, the presence of Ca^{++} during the reactions may result in incomplete degradation of the pectic substances (Solms 1954). These facts mean that the inhibitory effect of Ca^{++} may just as well be a substrate effect as an inhibition proper of the enzyme.

PE from higher plants demethoxylates polygalacturonic acid relatively independently of the molecule size, though the methyl-esters of mono-, di-, and tri-galacturonic acid were not attacked. The activity rate of PE depends on the distribution of the methoxyl groups along the pectin chain - probably in such a way that a methoxyl group neighbour to two carboxyl groups is more easily hydrolyzed than when placed in other combinations. This may be the reason why orange PE left 10% of the methylated carboxyl groups unhydrolyzed (Solms & Deuel 1955).

Sanner, Kovács-Proszt & Vas (1972) found that the activity of purified t-PE increased with the Ca^{++} concentration up to about 2.5×10^{-2}M at pH 7.5 with 0.1M phosphate buffer. Further, at lower Ca^{++} concentrations, or in the absence of Ca^{++}, an apparent stimulation of the PE activity was observed at low doses of γ-irradiation, and the radiation-induced inactivation of the enzyme was considerably reduced. Up to 2M NaCl made the t-PE more resistant to irradiation.

Tomatoes contain a pectin-PG different from that of molds (cf., e.g., McColloch & Kertesz 1948). t-PG degrades pectin down to 40–45% of that theoretically possible (Ozawa & Okamoto 1955a, b). The addition of more tomato enzyme did not change this, but if PG from molds was added instead, the hydrolysis became complete when calculated as reducing groups. When the enzyme preparation was heated it was shown to consist of both a heat labile and a heat resistant factor. Both factors had an optimum at pH 4.5 and were inactivated by Ca^{++}-ions.

The activity of t-PG is inhibited by Ca^{++}-ions like that from microorganisms (McClendon & Somers 1960).

Patel & Phaff (1960) studied the hydrolysis of polygalacturonic acid and various oligo-galacturonides using purified t-PG preparations almost free of t-PE but probably containing two enzyme components. The preparation caused a random hydrolysis down to mono-galacturonic acid, but with a much higher rate for hydrolysis of the polymers than for the lower oligomers. If the rate of hydrolysis of polygalacturonic acid is set at 100, the

rate for tetra-, tri-, and di-galacturonic acid was 7.0, 1.58, and 1.05, respectively. Hydrolysis of the polymer had pH optima at 2.5 and 4.5, that of the trimer and tetramer at pH 3.5 and 4.5, and that of the dimer at pH 4.4. According to the review of Rombouts & Pilnik (1972), only endo-PG is found in tomatoes.

Finally, it is worth noting the PG induction at the interaction between nodule bacteria (*Rhizobium* spp.) and their respective hosts. The nodule bacteria do not produce pectolytic enzymes in culture, but both PE and PG occur as exudates from nodulated legumes. PE also occurs as exudate from non-inoculated plants, whereas the PG only exudates from plants with nodule bacteria able to infect them. Furthermore, the PG is produced when cell-free preparations of polysaccharide from nodule bacteria are added to the root medium of an appropriate host. This means that PG production is induced in the host legume by specific polysaccharides from infective nodule bacteria (Nutman 1965).

Albersheim & Killias (1962) briefly mention the isolation of a pectin lyase from pea seedlings and note that it apparently behaves physically like that of *Aspergillus niger*. Other examples of the existence of this enzyme in higher plants are not found in scanning the literature.

The significance of pectolytic enzymes in higher plants has been under discussion for many years (Matus 1948) and much has still to be elucidated.

9.1.4. Animals

In the snail (*Helix pomatia* L. and others, Matus 1948, Holden 1950a, b) the intestinal fluid contains a PG with an optimum pH at 4-5, but broader than that for the activity of fungal PG. The snail PG is inhibited by high salt concentrations and Ca^{++}-ions are inhibitory in concentrations as low as 0.5×10^{-3}M. The activity on plant tissue increases when Ca^{++} is removed.

The plant bug, *Pyrrhocoris apterus* L., is found to produce an exo-PG releasing only galacturonic acid from pectic acid and oligomers (Courtois, Percheron & Foglietti 1968), but also other insects display pectolytic activity (cf. Bateman & Millar (1966) for further references on the pectolytic activity of insects).

Mah & Hungate (1965) showed that the rumen ciliate, *Ophryoscolex purkynei* Stein, produces an endo-pectate lyase releasing di- and trigalacturonic acid when degrading pectic acid. Other

protozoa are supposed to play a role in retting plant material (cf. Bateman & Millar 1966).

It has been reported that phytonematodes exhibit different pectolytic activities including PE, PG, and pectin lyase. For example, endo-PG and endo-pectin lyase are found in aqueous extracts from attacked onions, but not in the healthy plants. This was taken as an indication of the production of the enzymes by the nematodes. However, it is still doubtful whether the enzymes are produced by the nematodes themselves (Dropkin 1976).

Earth-worm excrements should exhibit a very high pectolytic activity (Matus 1948), but in the present author's opinion it remains unsolved whether this activity is produced by the animals themselves.

9.1.5. Commercially Prepared Pectolytic Enzymes

The importance of the methoxyl content of the pectin depends on the type of PG in question. For PMG a high content is preferred. For PG the activity decreases with increasing methoxyl content. Therefore, PE activity affects these polygalacturonase types in opposite ways. Jansen & MacDonnell (1945) have shown how the activity of a PG (Pectinol 100D) - probably endo-pectate PG as only the parts of the pectin molecule with free carboxyl groups were hydrolyzed - decreased with increasing methoxyl content, and Jansen, MacDonnell & Jang (1945) how a decreasing ratio of PG/PE enhanced hydrolysis by PG. At a ratio of 0.2, the demethoxylation was rapid enough to give a hydrolysis close to that of the action on pectic acid. Vice versa, the PG also enhanced the activity of the PE that is inhibited in the presence of pectic acid. The presence of Ca^{++} did not result in precipitation of Ca-pectate because of the rapid hydrolysis by the PG. The PE used for these experiments originated from orange fruits.

In this connection it should be mentioned that the influence of PE on the enzymatic breakdown of the molecular chain of pectin was shown as early as in 1899 by Bourguelot (Matus 1948).

Holden (1950c) incubated tobacco leaf fibre with purified, commercial, fungal PG (Pectinol 45AP), free of PE, at pH 4.5 in a Na-acetate buffer and found a liberation of carbohydrate from the fibre parallel to the activity of the enzyme. $CaCl_2$ concen-

trations up to about 0.25×10^{-3}M stimulated the activity whereas higher concentrations were progressively inhibitory. Thus tobacco fibre that had taken up the maximum possible amount of calcium was scarcely attacked by the PG. NaCl proved stimulatory in concentrations up to about 0.1M, and higher concentrations reduced the activity (see Figures 55 and 56). PE activity in the presence of calcium may after all affect the activity of PG, for which reason studies of the properties of PG proper cannot be made in the presence of PE. In this connection it should be mentioned that Rahman & Joslyn (1953) tested three PE-free purified PG's from commercial sources for several properties and found them only appreciably different in their pH optimum for activity.

Calcium was easily removed by ion exchange, and consequently oxalates are of importance for the removal of Ca^{++} from the cell walls.

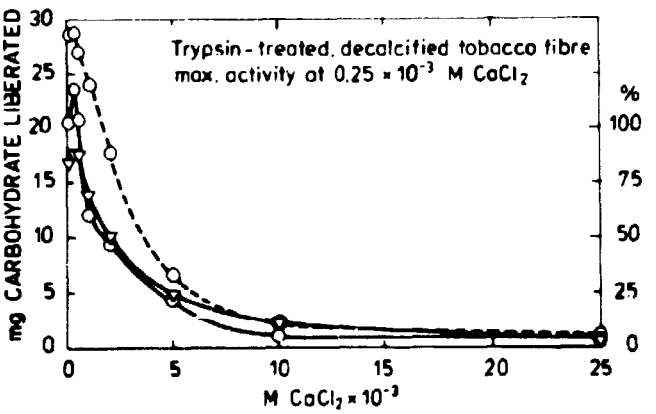

Figure 55. The effect of $CaCl_2$ on the percentage of polygalacturonate lost from tobacco-leaf fibre after 16 hours of treatment (▽) with a commercial polygalacturonase (Pectinol 45 AP) compared with the loss of carbohydrate after 1 (—○—) and 16 hours (- -○- -). (Drawn after Holden 1950, Table 6).

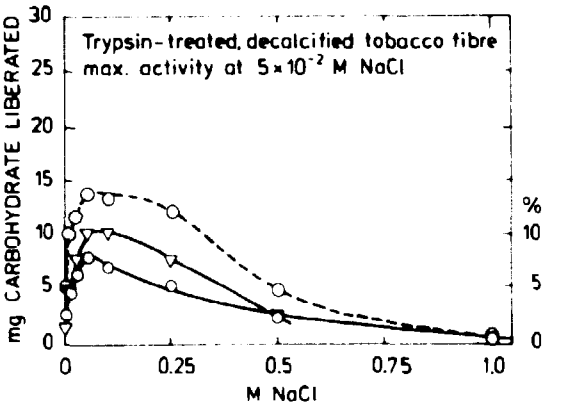

Figure 56. The effect of NaCl on the percentage of polygalacturonate lost from tobacco-leaf fibre after 17 hours of treatment (▽) with a commercial polygalacturonase (Pectinol 45 AP) compared with the loss of carbohydrate after 1 (—○—) and 17 hours (--○--). (Drawn after Holden 1950, Table 5).

9.2. Summary

The appendix, that reviews the pectolytic activity of a large number of organisms, is given in order to show the importance, uniformity and distribution of pectolytic enzymes in nature and the relation to the work presented above.

The very large amount of details from the investigations of all these organisms constitute a good, but rather difficult, basis for establishing some general conclusions on the pectolytic activity.

Pectinesterase is widely distributed in fungi, bacteria, and higher plants. It may or may not be produced adaptively, or production is just strongly stimulated by pectic substances. In some cases, more than one PE was found, e.g. in *Sclerotinia fructigena* and apples. If the PE's from fungi and bacteria are not identical, there is at least a pronounced similarity between them and between those from higher plants, but there is a distinct difference between the two groups. Apart from a questionable microbial PE with an optimum at pH 8-9 or higher, where also the saponification is active, the optimum for the fungal and bacterial

PE's lies at pH 5-5.5 or lower, whereas plant PE's have an optimum at about pH 7. The plant PE's are fixed to plant cell walls from where they may be liberated by NaCl, which is also necessary for their activity. Microbial PE is free and independent of NaCl; a property regarded as essential for its activity in the pathogenesis. Further, the microbial PE's are relatively temperature sensitive and sensitive to the general enzyme inhibitors, but very resistant to detergents, whereas plant PE's behave oppositely.

As far as the present author is aware, the effect of calcium on the microbial PE activity has only been mentioned in a single paper before. In the present treatise it is stated that Ca^{++}-ions enhance the initial rate of activity of *B. cinerea* and *S. sclerotiorum* PE and bring the reaction to a stop at a lower level of total hydrolysis. It has been stated that the rate of activity of PE depends on the distribution of the methoxyl groups in the molecular chain and if they are neighbours to one or two carboxyl groups. The effect of calcium in this connection may be that it blocks the free carboxyl groups.

In connection with other pectolytic enzymes, PE acts synergistically with those that have higher activity on pectate or low methylated pectins and antagonistically with those that are most active on highly methylated pectins. This is partly analogous to the synergistic effect of oxalic acid that removes calcium and liberates the carboxyl groups, and in contrast to the effect of calcium that blocks the carboxyl groups. Although PE in itself is without a tissue-macerating effect, it may greatly influence the activity of the other pectolytic enzymes, depending on the type and interacting substances present.

The polygalacturonases are very widely distributed though possibly more so in fungi than in bacteria, and they are also found in plants and animals (snails, plant bugs, and phytonematodes). They may or may not be produced adaptively, or their production may be stimulated in the presence of pectic substances, their degradation products, and chemicals such as asparagine. Further, some organisms produce these enzymes both in vivo and in vitro, whereas others only produce them in the host plants. In some cases polygalacturonases are produced in great amounts on artificial media, while the same enzymes are hardly detectable in attacked host tissue. The PG's exist in forms that at-

tack the molecular chain from the end (exo), or that attack more or less randomly within the molecule (endo). These enzymes may be divided up into some that prefer pectin (PMG) as substrate, others that prefer pectate (PG), and still others that are equally active on pectate and pectin.

Not only may the amount of a pectolytic enzyme produced differ from strain to strain, but the single type of enzyme may be produced in biochemically different components in the same organism. Some organisms produce a few pectolytic enzymes whereas others, e.g. *Aspergillus niger*, *Botrytis cinerea*, *Verticillium albo-atrum*, and *Erwinia chrysanthemi*, produce several.

The vast majority of the polygalacturonases have an optimum at about pH 5 or below. Only one obviously has an optimum at pH 8-9 (*Aphanomyces cuteiches*). There are several other cases in which an enzyme in one or another character deviates from the general behaviour of pectolytic enzymes.

Calcium-stimulated polygalacturonases have only been reported in *Thielaviopsis basicola* and *Verticillium albo-atrum*, and these cases need to be verified. All other papers dealing with the effect of calcium on the activity of these enzymes state that Ca^{++}-ions are strongly inhibitory; only the present author seems to have studied the calcium effect under the simultaneous action of other pectolytic enzymes in closer detail.

PE and oxalic acid may or may not interact and affect the effect of calcium depending on whether polygalacturonases or polymethylgalacturonases are the enzymes in question.

In work on *Rhizopus stolonifer*, anions such as NO_3^-, Cl^-, and SO_4^{--} were stated to be inhibitory to PMG.

The lyases are widely distributed too, but it is unknown if they are distributed to the same extent as the polygalacturonases, because only a smaller number of organisms was examined. These enzymes exist in different forms similar to those of the polygalacturonases, and their production may be adaptive or stimulated by the presence of pectic substances and their degradation products.

The lyases have an optimum pH at at 8-9 or above, and their activity is dependent on the presence of about 10^{-3}M Ca^{++}-ions, for which reason it may be completely prevented by EDTA. A few reports refer to a higher optimum for Ca^{++}-ions. Besides the present report, a few papers report a weakly depressive effect

on lyase activity at higher and increasing calcium concentrations.

The survey shows a great diversity of pectolytic enzymes. Some organisms have one set, others another, but always in such a way that the pectic substances can be decomposed under any conditions.

Some sets of enzymes may be inhibited by calcium under certain conditions, under which other sets are hardly influenced or are even stimulated. Thus the effect obtained after the application of calcium cannot be predicted unless the pectolytic activity of the pathogens in question is known. This general statement may also apply to carrots.

The interactions between pectolytic enzymes from one or more organisms may be synergistic or antagonistic, depending on their combinations and on the composition of the pectic substance they affect. In some cases such interactions have been shown also to include the pectolytic enzymes of the host tissue, e.g. the enzymes of the kaki fruits (persimmons) on attack by *Colletotrichum gloeosporioides*. In other cases, the host tissue contains substances inhibitory to pectolytic enzymes, e.g. compounds produced in apples by the activity of polyphenoloxydase or comparable cases in sweet potatoes and a protein from bean hypocotyls that agglutinates with the PG of *Colletotrichum lindemuthianum*.

The oligomers left by the endo-pectolytic enzymes may be further degradated by the exo-types of pectolytic enzymes or by special oligogalacturonases. The reaction products may act as inhibitors of further degradation. In other cases they possibly contribute to obstruct the flow in the vessels and thereby to cause a water shortage. In some cases, accordance has been observed between the pathotype and the production of PG or oxalic acid, but in other cases no relation was found between pectolytic activity and the ability of the organisms to establish an attack. Further the production of pectolytic enzymes is not limited to plant pathogens.

Ever since de Bary (1886) observed in the microscope that compounds produced by *Sclerotinia sclerotiorum* not only caused maceration of the plant tissue, but also made the cells unable to plasmolyze, which indicates cell death, the toxic agent has been a matter of discussion. It is repeatedly stated how endo--polygalacturonases and endo-lyases may directly cause cell death,

but that their ability to do so depends on the turgidity of the cells, whereas the exo-forms of these enzymes and the pectin-esterase exhibit no killing activity. However, any enzyme or other agent, e.g. oxalic acid, that acts synergistically with the endo-pectolytic enzymes indirectly increases the rate of killing, and any agent acting in the opposite manner protects the cells and the tissue. So does calcium.

10. GENERAL CONCLUSIONS

1. Irradiation is fully explained as an effective method to induce sprout and/or growth inhibition in carrots, potatoes, onions, and mushrooms.

2. Partial irradiation, e.g. limited to the top-end of carrots, has a good sprout inhibitory effect, but it may initiate an uncontrolled growth in the non-irradiated growing points in the lateral root traces and at the root-end, especially when stored under wet conditions. Such uncontrolled growth may have a tumorous appearance. It is, however, secondary and no special effect of irradiation as it may also be induced by mechanical injury to the top-end.

3. The effect of radiation is an inhibition of the formation of new cells, thus the formation of new periderm cells is inhibited simultaneously with the inhibition of sprouting. This leads to a loss of wound-healing capacity, though suberization of existing cells exposed in the wounds may continue in potatoes.

4. Irradiation increases the permeability of the plant tissues, which implicates a degradation of pectin in the middle lamellae and a release of calcium. These effects are measurable already at sprout inhibitory doses.

5. Irradiation disturbs the physiology of the products.

6. All these effects are analogous to those of the pathogens that attack vegetables. It is therefore supposed that these effects constitute the basis for the easier access of microorganisms to irradiated products.

7. This leads to the conclusion that doses of irradiation should be kept at the absolute minimum necessary for the purpose. This dose depends on the condition of the product and differs from one type of product to another. For potatoes and onions in deepest dormancy it is 8-10 krads and 6 krads, respectively. In carrots, 12 krads are needed to reduce top sprouting satisfactorily, and 200 krads are needed for a week's prolongation of the shelf-life of mushrooms without considerable pileus expansion and stipe

elongation.
The intensified attack by microorganisms may be reduced in proportion to the amount of irradiated tissue by means of partial irradiation.

8. $CaCl_2$ treatment may to some extent compensate for the irradiation-induced degradation of the plant tissue components as it enriches the tissue with calcium, hardens the tissue and reduces its permeability. At the same time calcium has an inhibitory effect on microbial attacks, both on the non-irradiated and on the irradiated tissue, but this inhibitory effect is incomplete and seems primarily exhibited in wounded products.

9. A closer examination of the effect of calcium on microbial attacks using disks of carrot as measuring objects showed that *Botrytis cinerea* and *Sclerotinia sclerotiorum* produce both thermolabile and thermostable agents with a tissue-macerating or softening effect. Both these organisms are strongly inhibited in the presence of calcium. The thermostable component has an effect similar to the effect of treatment of the tissue with oxalic acid.

10. In culture filtrates from *B. cinerea* the thermolabile agents comprise pectinesterase, polygalacturonase and pectin lyase, whereas those from *S. sclerotiorum* contain pectinesterase, polygalacturonase and little or no pectin lyase. The thermostable agent from both fungi is identified as oxalic acid, but *S. sclerotiorum* produces much more of the acid and at a higher rate than does *B. cinerea*.

11. The polygalacturonase from *B. cinerea* is more active on pectic acid than on pectin, whereas that from *S. sclerotiorum* is equally active on pectic acid and pectin.

12. The aim of the investigations with calcium was to elucidate its possible use as a protective measure in common practice against the intensified microbial attacks on irradiated products. Therefore the studies were concentrated on the simultaneous action on and interaction with pectic substances of the pectolytic enzymes from the two fungi, because the enzymes occur in culture filtrates and in attacked products in nature; although, according to the litera-

ture, the pure enzymes of these fungi are not among those most extensively studied.

13. The rate of hydrolysis caused by pectinesterase is enhanced by the addition of NaCl or $CaCl_2$ to the reaction mixture, but for the same ion strength the increase is much the highest for the addition of calcium. However, the level of total hydrolysis in the presence of NaCl and without salt added - which is equally high - is considerably higher than when $CaCl_2$ is present, and an increasing concentration of calcium results in a further decreased level of total hydrolysis.

14. The activity of polygalacturonase is strongly and progressively inhibited by increasing concentrations of $CaCl_2$.

15. Pectin lyase activity is dependent on the calcium ion. The addition of small amounts of $CaCl_2$ stimulated the lyase activity, and increasing concentrations of $CaCl_2$ only had a weak inhibitory effect.

16. Pectinesterase and polygalacturonase exhibit optimum activity about pH 5, whereas pectin lyase has optimum at pH 8.5. All three enzymes have a considerable activity at pH 6-7, which is the pH of many healthy plant tissues.

17. Oxalic acid (oxalate ion) softens the tissue by removing calcium ions from the pectin in the middle lamellae. In this respect it behaves analogously to irradiation. The removal of calcium may enhance the activity of polygalacturonase, which means that the effect is more than additive. Thus, oxalic acid and polygalacturonase act synergistically. On the other hand, oxalic acid may reduce the activity of pectin lyase because the activity of this enzyme depends upon calcium ions and is stimulated by a small increase in the concentration above this level.
Further, it is possible that the removal of calcium reduces the reaction rate of pectinesterase.
Finally, oxalic acid reduces the effect of calcium in proportion to the concentration and obviously has no effect on the enzyme activity proper.

18. A survey of the literature showed that various pectolytic enzymes are widely distributed among organisms in nature

and not confined to the plant pathogens, and some pathogens show very little or no pectolytic activity. The survey also showed that the spectrum of pectolytic enzymes covers the decomposition of all kinds of pectic substances under any condition.

19. The investigations showed that calcium has a compensatory effect of the irradiation-induced degradation of plant tissues (cf. point 8), and that it is very important during pathogenesis in connection with the activity of the pectolytic enzymes. In spite of this diversity of positive effects, calcium cannot be used as a general protective measure in common practice because pectolytic activity is not of importance during pathogenesis for all pathogens, and because the calcium ion is not very inhibitory to all pectolytic enzymes - under some conditions it may even stimulate the enzyme activity. Therefore the protective effect of calcium will not always be expressed to the same extent during storage, as it will depend on the conditions and on which organisms constitute the majority of the pathogens. Therefore, $CaCl_2$ is unusable as a protective measure against storage rot in general.

20. The significance of the increased susceptibility to rotting depends on several parameters which, if taken into account, may keep the radiation-induced higher incidence of rot at a low level, when compared with the advantages of sprout-inhibition. Little and careful handling, and the use of healthy, first-class, full-grown products, given a reasonable curing or wound-healing period before and/or after irradiation, will reduce storage rot. On this basis it is possible to keep potatoes and onions in first-class condition for half a year or more without any sprouting and with little rot. Radiation-induced sprout inhibition may also be used for carrots when they are removed from cold storage for keeping on the shelf at the greengrocer, or for long transport, but after a month or so the rotting starts to increase above the level of the non-irradiated carrots. Irradiation prolongs shelf-life of mushrooms by five days at $10^{\circ}C$, and the prolongation increases at lower storage temperatures.

21. The tendency of potatoes to develop internal discoloration when grown under unbalanced nutritional conditions may be enhanced after irradiation. In irradiated onions, the sprout buds may be discoloured if the bulbs are stored for several months at too high temperatures. The skin colour of irradiated mushrooms is generally better during storage than that of the non-irradiated mushrooms, if not packed too tightly. Internal discoloration also occurs in mushrooms, but it depends on physiology and packing method as well as on irradiation.

22. Radiation-induced sprout and growth inhibition hardly changes the taste and texture of vegetables apart from an induced, transitory, sweet taste in potatoes.

23. According to the literature, very extensive wholesomeness studies of irradiated vegetables have never revealed any adverse effect of irradiation.

24. All the parameters mentioned above must be taken into account when considering the commercialization of irradiation for growth and sprout inhibition in vegetables. Further, the question of economy must be considered, not only because of technical reasons and because the irradiation plant must be in operation throughout the whole year, but also because the products should be grown in the immediate vicinity of the plant in order to avoid long transport. Finally, there is an emotional and psychological barrier to be overcome before irradiated products win public acceptance.

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Dansk resumé

Der er gennemført en række forsøg, som har fastslået behandling med ioniserende stråler som en effektiv metode til hæmning af spiring og vækst i kartofler, løg, gulerødder og champignon. Forsøgene har imidlertid også vist, at stråledoserne må holdes på det mindste, der er nødvendig til formålet for at undgå uønskede bivirkninger. Disse doser er for kartofler og løg i dyb spirehvile henholdsvis 8-10 krad og 6 krad. For gulerødder, som er uden egentlig spirehvile, er 12 krad nødvendig for at opnå en tilfredsstillende hæmning af topspirerne, medens champignon må bestråles med 200 krad for at forlænge opbevaringen med en uge uden nævneværdig vækst af stokken og åbning af hatten.

Beskyttelse af gulerødderne, så kun topenden bliver bestrålet, giver god spirehæmning og reducerer den forøgede modtagelighed for råd, men samtidig resulterer det i en ukontrolleret, sekundær svulstagtig vækst i den ubestrålede ende af gulerødderne, når de opbevares under fugtige forhold. En lignende virkning kan fremkaldes på ubestrålede gulerødder ved bortskæring af topenden med en kniv.

Kartofler, løg og gulerødder har en forøget modtagelighed for råd efter bestråling med spirehæmmende doser, hvilket hænger sammen med de såringer og stød, som produkterne kommer ud for under optagning og håndtering, idet sårhelingsprocessen hæmmes, ligesom det er tilfældet med spiringen. Sårheling før og efter bestrålingen kan fjerne problemet i kartofler, fordi de har en udtalt evne til at danne sårperiderm, og fordi korkdannelsen i de blottede celler i sårene er i stand til at fortsætte efter bestrålingen, hvilket giver nogen beskyttelse. En lignende sårheling kan ikke anvendes til gulerødder, dels fordi sårhelingsevnen er mindre eller for langsom, dels fordi overfladen af gulerødderne mister turgesens eller tørrer ud, hvilket resulterer i forøget modtagelighed for råd. Den nærmere baggrund for den forøgede modtagelighed for råd efter bestråling med spirehæmmende doser og muligheden for at kompensere for den stråleinducerede skade på vævet er undersøgt med gulerødder som måleobjekt på grund af deres udtalte modtagelighed for råd efter bestrålingen.

Patogenerne forøger plantevævets permeabilitet foran væksten under patogenesen, og da bestrålingen har en analog virk-

ning, kan dette være medvirkende til at lette patogenernes adgang.

Bestrålingen øger calciums bevægelighed i plantevævet; sandsynligvis først og fremmest af det, der er bundet til pectinstofferne i midtlamellerne. Derved bliver pectinet lettere tilgængeligt for patogenernes angreb. Samtidig virker bestrålingen blødgørende på vævet. Behandling med calcium i form af $CaCl_2$ øger plantevævets fasthed, reducerer dets permeabilitet og kan kompensere for calciumtabet i det bestrålede væv, hovedsagelig ved et overskud af calcium i sårene. Det er vist, at dette giver en betydelig beskyttelse af sårede gulerødder under opbevaringen, uanset om de var bestrålede eller ikke.

Botrytis cinerea og *Sclerotinia sclerotiorum* var blandt de mest udbredte og alvorlige patogener i de bestrålede gulerødder. Angreb af *B. cinerea* forøgedes ved udtørring af gulerøddernes overflade, medens angrebet af *S. sclerotiorum* reduceredes under sådanne forhold. Kulturfiltrater fra disse organismer viste en kraftig macererende aktivitet på gulerodsvæv. Forskellen mellem virkningen af kulturfiltraterne fra de to organismer viste sig at skyldes en termostabil faktor, der dannedes i relativt store mængder af *S. sclerotiorum*. Denne faktor havde en abrupt macererende eller blødgørende virkning, som ophørte efter omkring en times behandling på samme måde som det var tilfældet ved forsøg med oxalsyre.

Tilstedeværelse af calcium hindrede eller reducerede i høj grad den macererende virkning, og det fjernede totalt virkningen af den termostabile faktor.

Nærmere undersøgelser viste, at *B. cinerea* danner tre pectinnedbrydende enzymer, pectinesterase, polygalacturonase, der er mere aktiv på pectinsyre end på pectin, og lyase, som er meget aktiv på pectin og kun svagt aktiv på pectinsyre samtidig med, at der sker en langsom produktion eller ophobning af oxalsyre i vækstsubstratet. *S. sclerotiorum* danner pectinesterase, polygalacturonase lige aktiv på pectin og pectinsyre, men derimod ingen eller yderst lidt lyase samtidig med, at der sker en hurtig produktion eller ophobning af store mængde oxalsyre i vækstsubstratet. To andre gulerodspatogener, *Chalaropsis thielavioides* og *Mycocentrospora acerina*, dannede små mængder pectolytiske enzymer, som kun kunne påvises gennem ophobning af deres reaktionsprodukter i vækstsubstraterne.

Aktiviteten af de enkelte enzymer blev undersøgt under samtidig indvirkning af de øvrige, så det opnåede resultat havde mulighed for at ligne det, der finder sted i naturen. Under disse betingelser havde calcium en forøgende virkning på aktiviteten af pectin methyl esterase, men den standsede på et lavere niveau af total hydrolyse end ved tilstedeværelse af natrium ioner eller uden salte. Aktiviteten af polygalacturonase reduceredes stærkt af calcium, hvorimod lyaseaktiviteten øgedes svagt af små mængder calcium og reduceredes svagt med stigende koncentration af calcium i reaktionsvæsken. Oxalsyren reducerede virkningen af calcium i forhold til koncentrationen, men det havde ingen indflydelse på selve enzymaktiviteten.

De forskellige pectolytiske enzymer er vidt udbredt blandt organismerne i naturen og ikke alene begrænset til plantepatogenerne. Desuden viser nogle patogener kun svag eller ingen pectolytisk aktivitet. På den baggrund, på grund af at der forekommer pectolytiske enzymer, så nedbrydning af pectinstoffer kan finde sted så at sige under enhver betingelse, og fordi calcium ikke hæmmer alle pectolytiske enzymer, kan det ikke forventes, at den klart beskyttende virkning af calcium altid vil komme til udtryk i samme grad under produkternes opbevaring. Det vil afhænge af de ydre betingelser og af de organismer, der udgør hovedparten af patogenerne i det pågældende tilfælde. Det er desuden slået fast, at den beskyttende virkning af calcium reduceres parallelt med reduktionen af såringerne, hvorfor hovedparten af virkningen menes knyttet til ophobningen af calcium i sårene. Det kan således ikke generelt anvendes som middel til bekyttelse mod opbevaringsråd.

En ulempe for brugen af bestråling som spire- og vækshæmmende middel er også den indre misfarvning, der kan forekomme i kartofler, løg og champignon. Denne virkning kan dog gøres ubetydelig ved anvendelse af gode dyrknings- og opbevaringsbetingelser og ved pakning, så der er god luftudskiftning uden udtørring af produkterne, først og fremmest for champignon.

Spire- og vækshæmmende stråledoser ændrer næppe produkternes smag og fasthed bortset fra en forbigående sødere smag i kartofler.

Når der kun anvendes 1. klasses produkter, og ulemperne imødegås, vil bestråling kunne anvendes til hæmning af spiring i kartofler, løg og gulerødder samt til hindring af fortsat udvikling eller vækst i champignon.

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